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The "Turkish Journal of Obstetrics and Gynecology" is the official publication of the Turkish Society of Obstetricians and Gynecologists. The journal is published quarterly (March, June, September and December) in English and publishes original peer-reviewed articles, reviews, case reports and commentaries in the fields of gynecology, gynecologic oncology, endocrinology and reproductive medicine and obstetrics. The journal gives publication priority to original research articles over case reports. Reviews are considered for publication only if they are prepared by authors who have at least three published manuscripts in international peer-reviewed journals on the topic of the review and these studies should be cited in the review. Otherwise only invited reviews will be considered for peer-review from qualified experts in the area.

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PRISMA for preferred reporting items for systematic reviews and meta-analyses (Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group. Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 2009; 6(7): e1000097.) (http://www.prisma-statement.org/),

STARD checklist for the reporting of studies of diagnostic accuracy (Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig LM, et al, for the STARD Group. Towards complete and accurate reporting of studies of diagnostic accuracy: the STARD initiative. Ann Intern Med 2003;138:40-4.) (http://www.stard-statement.org/),

STROBE statement-checklist of items that should be included in reports of observational studies (http://www.strobe-statement.org/),

MOOSE guidelines for meta-analysis and systemic reviews of observational studies (Stroup DF, Berlin JA, Morton SC, et al. Meta-analysis of observational studies in epidemiology: a proposal for reporting Meta-analysis of observational Studies in Epidemiology (MOOSE) group. JAMA 2000; 283: 2008-12).

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A separate title page should list;

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- A short title of no more than 50 characters, including spaces, for use as a running foot.

- All author name(s), institutional, corporate, or commercial affiliations, and up to two major degree(s).

- Corresponding author's name, address, telephone (including the mobile phone number), fax numbers and e-mail address (the corresponding author will be responsible for all correspondence and other matters relating to the manuscript).

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The precis is a one-sentence synopsis of no more than 30 words that describes the basic findings of the article. Precis sample can be seen below:

'Using a 45 point questionnaire, we have evaluated the trend of Robotic surgery training in the gynecologic surgery fellowship programs across the nation'.

Abstract

All manuscripts should be accompanied by an abstract. All information in the abstract should be consistent with the information in the text, tables, or figures. Avoid use of commercial names in the abstract. Original research reports should have a structured abstract of no more than 250 words, using the following headings:

· Objective: Main question, objective, or hypothesis (single phrase starting with, for example, "To evaluate..." or "To estimate." [never start with "To determine."]).

· Materials and Methods: Study design, participants, outcome measures, and in the case of a negative study, statistical power.

· Results: Measurements expressed in absolute numbers and percentages, and when appropriate indicate relative risks or odds ratios with confidence intervals and level of statistical significance; any results contained in the abstract should also be presented in the body of the manuscript, tables, or figures.

· Conclusion: Directly supported by data, along with clinical implications.

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A structured abstract is not required with review articles and case reports.

Keywords

Below the abstract provide 3 to 5 keywords. Abbreviations should not be used as keywords. Keywords should be picked from the Medical Subject Headings (MeSH) list (www.nlm.nih.gov/mesh/MBrowser.html).

Turkish abstracts should have keywords "Anahtar Kelimeler" picked from www.atifdizini.com under "Türkiye Bilim Terimleri" link.

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Table 1. Manuscript length at a glance

Article type	Abstract Length	Manuscript Word Count*	Maximum Number of Authors	Maximum Number of References$^{\Phi}$
Original Research	250 words	,500 words (~22 pages)$^{\Psi}$	NA	30
Case report	150 words	,000 words (~8 pages)	4	8
Systematic review	300 words	6,250 words (~25 pages)	4	60
Current commentary	250 words	,000 words (~12 pages)	4	12
Procedure and Instruments	200 words	,000 words (~8 pages)	4	10
Letters	NA	350 words	4	5

*Manuscript length includes all pages in a manuscript (ie, title page, abstract, text, references, tables, boxes, figure legends, and appendixes). $^{\Phi}$Suggested limit. $^{\Psi}$The Introduction should not exceed 250 words. ~approximately; NA, not applicable.

Original researches should have the following sections;

Introduction

State concisely the purpose and rationale for the study and cite only the most pertinent references as background. Avoid a detailed literature review in this section.

Materials and Methods

Describe the research methodology (the patients, experimental animals, material and controls, the methods and procedures utilized, and the statistical method(s) employed) in sufficient detail so that others could duplicate the work. Identify methods of statistical analysis and when appropriate, state the basis (including alpha and beta error estimates) for their selection. Cite any statistical software programs used in the text. Express p values to no more than two decimal places. Indicate your study's power to detect statistical difference.

Address "IRB" issues and participants informed consent as stated above, the complete name of the IRB should be provided in the manuscript. State the generic names of the drugs with the name and country of the manufactures.

Results

Present the detailed findings supported with statistical methods. Figures and tables should supplement, not duplicate the text; presentation of data in either one or the other will suffice. Authors should report

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outcome data as both absolute and relative effects since information presented this way is much more useful for clinicians. Actual numbers and percentages should be given in addition to odds ratios or relative risk. When appropriate, number needed to treat for benefits (NNTb) or harm (NNTh) should be supplied. Emphasize only your important observations; do not compare your observations with those of others. Such comparisons and comments are reserved for the discussion section.

Discussion

Begin with a description of what your study found in relation to the purpose or objectives as stated in the Introduction. State the importance and significance of your findings to clinicians and actual patient care but do not repeat the details given in the Results section. Limit your opinions to those strictly indicated by the facts in your report. Compare your finding with previous studies with explanations in cases where they differ, although a complete review of the literature is not necessary.

Study Limitations

Provide information on the limitations of the study. No new data are to be presented in this section. A final summary is not necessary, as this information should be provided in the abstract and the first paragraph of the Discussion. Although topics that require future research can be mentioned, it is unnecessary to state, "Further research is needed."

Conclusion

The conclusion of the study should be highlighted. The study's new and important findings should be highlighted and interpreted.

Conflict of Interest

Authors must indicate whether or not they have a financial relationship with the organization that sponsored the research.

The main text of case reports should be structured with the following subheadings:

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References are numbered (Arabic numerals) consecutively in the order in which they appear in the text (note that references should not appear in the abstract) and listed double-spaced at the end of the manuscript. The preferred method for identifying citations in the text is using within parentheses. Use the form of the "Uniform Requirements for Manuscripts" (http://www.icmje.org/about-icmje/faqs/icmje-recommendations/). If number of authors exceeds seven, list first 6 authors followed by et al.

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TURKISH JOURNAL OF OBSTETRICS AND GYNECOLOGY

EDITORIAL

Dear Colleagues,

TJOD journal, which is the media organ of the Turkish Society of Obstetrics and Gynecology, is hereby presented to you once again with its September issue containing high scientific content. We believe that the steady increase in the number of our monthly readings is the most significant parameter of the efforts made and the meticulousness of the evaluation process.

Dear colleagues, one of the most important indicators of our scientific activities as TJOD is surely the presence of our Turkish Journal of Obstetrics and Gynecology, which is now well accepted and respected on international platforms. In addition, as a society, we conducted our scientific activities with great devotion in the month of September. On the dates of September 7-10, 2022, we completed the "Master Class" courses organized by our Editor-in-Chief, Eray Calıskan, M.D. in Istanbul with great participation. We also held the National Congress of Gynecological Endoscopy on the dates of September 7-11 in Istanbul in collaboration with international speakers and with great participation. We have currently intensified our efforts with great excitement for the scientific program of the National Congress of Gynecology and Obstetrics, which will be held on the dates of May 17-21, 2023 in Cyprus.

Firmly believing that original scientific articles in the September issue of our journal will make a great contribution to the repertoire of knowledge of our colleagues, I am looking forward to meeting our colleagues who have devoted themselves to science in the December issue.

Ercan Yilmaz, MD
Co-Editor in Chief

Clinical Investigation / Araştırma

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Turk J Obstet Gynecol 2022;19:178-86

Effect of asymptomatic COVID-19 infection on the placenta in the third trimester of pregnancy: A prospective case-control study

Term gebeliklerde asemptomatik COVID-19'un plasentaya etkisi: Prospektif olgu-kontrol çalışması

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Abstract

Objective: To clarify the effect of asymptomatic coronaviruse disease-2019 (COVID-19) positivity on the placenta in the third trimester of pregnancy.

Materials and Methods: This prospective, case-control study included 30 pregnant women diagnosed with asymptomatic COVID-19 between April 30, 2021 and July 20, 2021 who delivered after the 34th gestational week, and a control group of 30 pregnant women without COVID-19, who delivered between April 2021 and July 2021, matched to the study group regarding age, gestational age and body mass index. Outcomes were compared in terms of demographic characteristics, serum blood outcomes, neonatal results, complications and placental histopathological findings.

Results: The mean age of the study population was 28.8 years and the mean gestational week was 38.2 weeks. The C-reactive protein levels (38.2 mg/L vs 5.8 mg/L, p=0.001) and ferritin levels (266.4 µg/L and 40.5 µg/L, p=0.001) were significantly higher in the COVID-19-positive pregnant women. The lymphocyte level was significantly higher in the non-COVID-19 pregnant women (p=0.040). Mural hypertrophy was determined at a significantly higher rate in COVID-positive pregnant women (83.3% vs 30.0%, p=0.001). Multivariate regression analysis showed that only COVID-19 positivity increased the presence of mural hypertrophy in pregnant women with asymptomatic COVID-19 (4.716-fold, 95% confidence interval=1.012-22.251).

Conclusion: The results of this study demonstrated that asymptomatic COVID-19 had no significant effect on pregnancy and neonatal complications. However, mural hypertrophy in the placenta was found at a significantly higher rate in pregnant women with asymptomatic COVID-19.

Keywords: Asymptomatic, COVID-19, mural hypertrophy, placenta, pregnancy

Öz

Amaç: Term gebeliklerde asemptomatik koronavirüs hastalığı-2019 (COVID-19) pozitifliğinin gebelik ve plasenta üzerindeki etkisini araştırmak amaçlandı.

Gereç ve Yöntemler: Bu prospektif, olgu-kontrol çalışmasına 30 Nisan 2021 ile 20 Temmuz 2021 tarihleri arasında asemptomatik COVID-19 tanısı konan ve 34. gebelik haftasından sonra doğum yapan gebeler ile COVID-19 olmayan 30 gebeden oluşan bir kontrol grubu dahil edilmiştir. Kontrol grubu ile çalışma grubu yaş, gebelik yaşı ve vücut kitle indeksi açısından eşleştirildi. Sonuçlar demografik özellikler, serum kan sonuçları, neonatal sonuçlar, komplikasyonlar ve plasental histopatolojik bulgular açısından karşılaştırıldı.

Bulgular: Çalışma popülasyonunun ortalama yaşı 28,8 yıl ve ortalama gebelik haftası 38,2 hafta idi. COVID-19 pozitif gebelerde C-reaktif protein düzeyi (38,2 mg/L vs 5,8 mg/L, p=0,001) ve ferritin düzeyi (266,4 µg/L ve 40,5 µg/L, p=0,001) anlamlı olarak daha yüksekti. COVID-19 olmayan gebe kadınlarda lenfosit düzeyi anlamlı olarak daha yüksekti (p=0,040). Mural hipertrofi, COVID-19 pozitif gebe kadınlarda anlamlı olarak daha yüksek oranda belirlendi (%83,3'e karşı %30,0, p=0,001). Çok değişkenli regresyon analizi ile asemptomatik COVID-19'lu gebe kadınlarda sadece COVID-19 pozitifliğinin mural hipertrofi varlığını anlamlı oranda artırdığı gösterildi (4.716 kat, %95 güven aralığı=1.012-22.251).

PRECIS: Asymptomatic COVID-19 positive 3rd trimester pregnant woman has vascular pathology that may complicate pregnancy in the placenta.

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Sonuç: Bu çalışmanın sonuçları asemptomatik COVID-19'un gebelik ve yenidoğan komplikasyonları üzerinde anlamlı bir etkisinin olmadığını göstermiştir. Bununla birlikte, asemptomatik COVID-19'lu hamile kadınlarda plasentada mural hipertrofi önemli ölçüde daha yüksek oranda bulundu.

Anahtar Kelimeler: Asemptomatik, COVID-19, mural hipertrofi, plasenta, gebelik

Introduction

The new coronavirus disease-2019 (COVID-19), which was first determined at the end of 2019, spread rapidly around the world within a few months and was declared a pandemic by the World Health Organisation on March 11, 2020 [1]. As little is currently known about COVID-19 in pregnancy, the consequences of infecting pregnant women with COVID-19 and the potential risks of vertical transmission have become a major concern. In a previous large series of studies, it has been shown that pregnant women aged 15-44 years were diagnosed with COVID-19 infection at a rate of 9%, which was higher than the rate of 5% in the general female population of reproductive age[2]. Cardona-Pérez et al.[3] reported that severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) real time-polymerase chain reaction (PCR) positivity was determined in 29% of pregnant women, of whom 86% were asymptomatic. In another study, it was reported that 5.7% females diagnosed with COVID-19 were pregnant and showing symptoms[4]. In two cohort studies in Turkey, universal screening was applied to all the patients who presented for childbirth, and asymptomatic SARS-CoV-2 carriers were determined at the rates of 7.7% and 1.4%[5,6].

Among these asymptomatic patients admitted for labor and childbirth, the global rates of SARS-CoV-2 positivity were reported to range between 0.45% and 19.9%[7]. Breslin et al.[8] observed that symptoms developed during labor or in the postpartum period in 71% of asymptomatic cases who presented for childbirth.

In a meta-analysis that included 42 studies of 438,548 pregnant women, COVID-19 was associated with an increased risk of preterm birth, pre-eclampsia, and stillbirth, compared with women who were SARS-CoV-2 negative during pregnancy[9]. In another study, Allotey et al.[10] emphasized that pregnant women with COVID-19 had significantly higher risks for preterm delivery, requirements for the intensive care unit and maternal death compared with pregnant women without COVID-19. Furthermore, Tasca et al.[11] claimed that high perfusion of the placenta with COVID-19 infection could increase the inflammatory response and oxidative stress, which could cause undesirable events in pregnant women.

The placenta may be affected by the virus in pregnant women with COVID-19 positivity. Although the probability of vertical transfer seems to be low, it has been indicated that the fetus can be potentially affected by the placenta infection[12]. Vertical transfer of SARS-CoV-2 determined in pregnancy has not yet been clarified and research on the effects of the viral pathogen on the placenta is ongoing.

Many studies have focussed more on examining the placental effects of COVID-19 in symptomatic pregnant patients and have attempted to explain the effect of this on the pregnancy. These studies have revealed some common morphologies histologically and there has been observed to be a predominance of vascular malperfusion (VM) and placental infection with histiocytic intervillositis increased perivillous fibrin, and villous trophoblast necrosis[13,14]. In contrast, there are very limited data related to how the effect is formed in asymptomatic COVID-19-positive pregnant patients.

This study aimed to clarify the effect of asymptomatic COVID-19 on the placenta by comparing the placentas of pregnant women with asymptomatic COVID-19 and pregnant women without COVID-19 in the third trimester.

Materials and Methods

This study was conducted as a prospective, case-control study. One of the primary study endpoints was the rate of placental vascular pathology, which was expected to be higher in asymptomatic COVID-19-positive pregnant women. With reference to a study by Jaiswal et al.[15], sample size to provide α 0.5, and power of (1-β) 80, was calculated as a minimum of 60 study participants (30 COVID-positive asymptomatic pregnant women during delivery and 30 COVID-negative pregnant women during delivery in the control group) to be sufficient to produce statistically significant results.

The COVID-19-positive group included asymptomatic pregnant women who were positive after the routine PCR test performed for patients who were hospitalized for delivery and who had no suspicious complaints. A control group was formed from patients who were admitted to the hospital for delivery within the same period with a negative routine PCR test, matched according to age, gestational week and body mass index (BMI). The patients in the control group were tested for antibodies (Weimi Diagnostic, Guangzhou Weimi Bio-Tech, Guangzhou, China) to exclude the possibility of having asymptomatic COVID-19 during pregnancy. Those with a negative result were deemed not to have had COVID-19 and were included in the control group.

Demographic and clinical information of the two groups was obtained from the hospital information system (PANATES® hospital information systems) and patient records. Ethics committee approval was obtained from the local "Medical Ethics and Institutional Review Board" with ID number E-48670771-514.10/136 in April 19, 2021, and the study was completed in accordance with Helsinki Declaration principles. All the pregnant women provided informed consent to participate in the study. The pregnant women diagnosed with asymptomatic COVID-19 and those without COVID-19 were accepted as

candidates for the study between April 30, 2021 and July 20, 2021, and those who delivered after the 34th gestational week were included in the study. Exclusion criteria were defined as a history of COVID-19 during pregnancy, the presence of multiple pregnancy, the presence of any chronic disease during pregnancy (neurological disease, heart disease, renal disease, immune disorder, etc.) or the use of drugs (not including pregnancy vitamin supplements and iron preparations) due to such diseases, a history or diagnosis of hypertension during pregnancy, diabetes mellitus, fetal growth retardation, infections (eg., toxoplasmosis, rubella, cytomegalovirus, herpes simplex), placental anomalies, vascular pathology determined on obstetric Doppler ultrasonography performed during pregnancy (abnormal pulsatility index, uterine artery notch etc.), placenta previa, fetal anomaly diagnosis, or incomplete data in the records.

The demographic characteristics of all pregnant women including age, gestational age (weeks), presence of gestational diabetes mellitus, maternal-obstetric complications such as amniotic volume disorders, intrapartum-postpartum adverse results, such as massive bleeding, new onset hypertension, dyspnea, nasal O_2 requirement, intensive care admission that may be related to COVID-19, BMI and laboratory results including C-reactive protein (CRP), ferritin level, white blood cell (WBC) level, neutrophil and lymphocyte levels were recorded. Neonatal data including the 5-min APGAR score, requirement for neonatal intensive care unit, pH of umbilical cord blood, and placental weight and diameter were also noted.

Histopathological Evaluation

All placental samples were stored in the pathology department and a single experienced gynecopathologist (A.Y.A.) performed all the histopathological and morphological analyses to minimize possible bias. The pathologist was blinded to clinical information, including the COVID-19 status. Placentas were fixed with 10% neutral buffered formalin and the Amsterdam Placental Workshop Group 2016[16] classification was used to determine macroscopic and microscopic lesions. Two full thickness sections from the umbilical cord insertion site and a total of twelve sections including placental membranes and umbilical cord, were taken for examination. The pathological findings examined included ectasia and thrombosis of the vascular cord, infarcts, retroplacental hemorrhage, chorangiosis, distal villous hypoplasia, accelerated villous maturation, decidual arteriopathy, thrombosis, avascular villi, villous stromal karyorrhexis, mural hypertrophy, stem villous obliteration, delayed villous maturation, funisitis, chorioamnionitis, villitis, and perivillous fibrin deposition.

Statistical Analysis

Data obtained in the study were analyzed statistically using the IBM SPSS Statistics Version 25 (NY, USA). Shapiro-Wilk test and Q-Q plot tests were performed as the normality test. The Independent t-test was used for comparisons between the groups of normally distributed variables, and the Mann-Whitney U test was used for not normally distributed data. The chi-square (χ^2) probability distribution was applied in analyzing categorical variables and Fishers' Exact probability test was used when the assumptions for conducting a chi-square test were not met. Quantities are shown as mean ± standard deviation. A logistic regression model was performed using odds ratios to investigate confounding factors in the observed association. The data were resolved at 95% confidence interval level and $p<0.05$ was considered statistically significant.

Results

According to the study design, 30 pregnant women with asymptomatic COVID-19 and 30 pregnant women without COVID-19 were enrolled in the study. The mean age of the study population was 28.8 years and the mean gestational week was 38.2 weeks. The mean placental weight and diameter was 587.5 g and 18.0 cm, respectively. Cesarean section (C/S) was performed in 39 (65%) patients. A total of 51 infants were born with a 5-min APGAR score more than 7, and the mean pH of umbilical cord blood 7.3. The demographic characteristics of the study population, serum blood outcomes, placental findings and neonatal results are summarized in Table 1.

Comparisons of the COVID-positive and COVID-negative pregnant women revealed that age, gestational age, BMI, WBC level, neutrophil level, 5-min. APGAR score, the pH of umbilical cord blood and maternal blood pressures were comparable (p=0.059, p=0.412, p=0.499, p=0.311, p=0.647, p=0.071, p=0.649 and p=0.404, respectively). Placental weight and placental diameter was similar between the groups (p=0.346 and p=0.930). CRP level (38.2 mg/L vs 5.8 mg/L, p=0.001) and ferritin level (266.4 µg/L and 40.5 µg/L, p=0.001) were significantly higher in the COVID-19-positive pregnant women. Lymphocyte levels were significantly higher in pregnant women without COVID-19 (p=0.040), but the neutrophil/lymphocyte ratio was not statistically significant (p=0.157) (Table 2). The requirement for maternal nasal O_2 was significantly higher in the COVID-19-positive pregnant women (p=0.024). Complications were detected in one pregnant woman without COVID-19 and in four pregnant women with COVID-19 (p=0.706). The types of complications are listed in Table 2.

In the pregnant women with asymptomatic COVID-19, mural hypertrophy (83.3%), distal villous hypoplasia (63.3%) and perivillous fibrin deposition (56.7%) were the most common histological findings and in pregnant women without COVID-19, perivillous fibrin deposition (76.7%), distal villous hypoplasia (40.0%) and infarcts (33.3%) were most common. Only mural hypertrophy was significantly common in COVID-19- positive pregnant women (83.3% vs 30.0%, p=0.001) (Figure 1). No statistically significant difference was determined between the groups with respect to the other pathological findings (Table 3). Univariate analysis revealed that higher CRP value and COVID-19 positivity was significantly common in pregnant

women with mural hypertrophy (p=0.014 and p<0.001) and increased the mural hypertrophy risk 1.066-fold and 11.667-fold, respectively. The multivariate regression analysis showed that only COVID-19 positivity increased the presence of mural hypertrophy in pregnant women with asymptomatic COVID-19 [4.716-fold, 95% confidence interval (CI)=1.012-22.251] (Table 4).

Discussion

This study aimed to investigate the effect of COVID-19 on the placenta by comparison with COVID-19-negative third trimester pregnant women. The study results showed mural hypertrophy to be significantly more common in the placenta of COVID-19-positive pregnant women. Although specific placental histopathological changes in patients infected with COVID-19 have been suggested in some previous studies, there have been no appropriate control groups in those studies[11,17,18]. The current study results showed vasculopathy in maternal-fetal placental vessels as a significant finding in the cohort of asymptomatic SARS-CoV-2-positive patients, which was consistent with the findings of some previous studies[19]. Additionally, it was determined that CRP and ferritin levels were significantly higher and lymphocyte levels were lower in pregnant women with asymptomatic COVID-19 positivity, as well as an increased need for maternal nasal O_2 during delivery and an increase in the rate of cesarean section delivery.

Table 1. Demographic data for all patients

	Mean ± SD/n (%)
Age (years)	28.8±5.9
Gestational age (weeks)	38.2±1.7
BMI (kg/m^2)	24.0±2.8
CRP (mg/L)	22.0±30.5
Ferritin (µg/L)	153.4±288.9
WBC (10^3/µL)	10.4±3.6
Neutrophil (10^3/µL)	8.0±3.2
Lymphocyte (10^3/µL)	1.8±0.7
Neutrophil/lymphocyte ratio	5.1±2.7
5-min. APGAR score	
≤7	9 (15.0%)
>7	51 (85.0%)
pH of umbilical cord blood	7.3±0.1
Insertion anomaly	3 (5.0%)
Placental weight (g)	587.5±119.3
Placental diameter (cm)	18.0±2.5
Type of delivery	
Vaginal delivery	21 (35.0%)
C/S	39 (65.0%)
Nasal O_2 requirement	6 (10.0%)
Gestational diabetes mellitus	1 (16.7%)
NICU requirement	9 (15.0%)

BMI: Body mass index, CRP: C-reactive protein, WBC: White blood cell, C/S: Cesarean/section, SD: Standard deviation, NICU: Neonatal intensive care unit

Angiotensin-converting enzyme 2 is expressed in the human placenta, primarily in syncytiotrophoblasts and cytotrophoblasts and secondarily in placental villi. It is also found in the arterial and venous endothelium of the umbilical cord, thereby rendering it theoretically possible that COVID-19 can spread to and affect the placenta from the mother[20]. Placental trophoblast atherosis represents a series of morphological spectra compatible with placental pathology and may be associated with different pathogenic mechanisms such as viral, or inflammatory responses related to trophoblast cell death and vascular remodeling. Mural hypertrophy can be evaluated as a histopathological result of vascular changes known as placental atherosis or VM[21].

In the present study, only mural hypertrophy was significantly common in the pregnant women with COVID-19, and asymptomatic COVID-19 positivity increased the risk of mural hypertrophy in the placental vascular bed 4.716-fold. Jaiswal et al.[15] compared the placentas of COVID-19-positive and COVID-19-negative women and showed that there were significantly higher maternal-fetal malperfusion changes in pregnant women with COVID-19. In contrast, Blasco Santana et al.[22] analyzed the placenta histology in 29 patients and concluded that COVID-19 did not change any histological properties of the placenta. Moreover, it was claimed in that study that the placenta formed a barrier against the spread of COVID-19. In the review, which evaluated the histopathological findings in 3rd trimester placentas with infected maternal SARS-CoV-2, evidence of fetal and maternal VM was reported at the rate of 35% (95% CI: 27.7%-43.0%) and 46% (95% CI 38.0%-54.0%) cases, respectively[23]. In a recent study by Patberg et al.[24] on asymptomatic pregnant women, VM rates were found to be higher than those of the control group (31.3% vs 3.6%, p<0.0001). Although these rates are lower than those of the current study cohort, they are compatible with this study. However, the reason for the lower rates could be attributed to the absence of blinding and control groups in most of the studies. The considerable variation in these studies limits the comparisons that can be made with the current study findings.

Theoretically, SARS-CoV-2 can affect placental development during pregnancy either directly through infection or indirectly through inflammation it elicits. Although recent studies seem to to support vertical transmission of the SARS-CoV-2 in contrast to previous studies, this feature has not yet been comprehensively described in the literature[25]. Additionally, the presence of SARS-CoV-2 RNA in placental tissue has still not been clearly demonstrated. In parallel with the findings of Patberg et al.[24], the present study results

Table 2. Comparison of demographic and clinical data between groups

	COVID (+) n=30	COVID (-) n=30	p-value*
Age (years)	30.3±5.7	27.4±5.9	0.059
Gestational age (weeks)	38.0±1.6	38.4±1.8	0.412
BMI (kg/m^2)	23.7±3.2	24.2±2.4	0.499
CRP (mg/L)	38.2±36.6	5.8±3.8	**0.001**
Ferritin (µg/L)	266.4±378.0	40.5±23.1	**0.001**
WBC (10^3/µL)	9.9±3.5	10.9±3.7	0.311
Neutrophil (10^3/µL)	7.9±3.0	8.2±3.5	0.647
Lymphocyte (10^3/µL)	1.6±0.7	1.9±0.5	**0.040**
Neutrophil/lymphocyte ratio	5.6±2.6	4.6±2.8	0.157
5-min. APGAR score			
≤7	7 (23.3%)	2 (6.7%)	0.071
>7	23 (76.7%)	28 (93.3%)	
pH of umbilical cord blood	7.3±0.1	7.3±0.1	0.649
Placental weight (gr)	572.9±100.0	602.2±136.1	0.346
Placental diameter (cm)	18.0±2.6	18.0±2.3	0.930
Type of delivery			
Vaginal delivery	6 (20.0%)	15 (50.0%)	**0.015**
C/S	24 (80.0%)	15 (50.0%)	
Fetal distress	6 (25%)	6 (40%)	0.478
Mother request	7 (29.1%)	1 (6.6%)	0.121
Previous cesarean section	5 (20.8%)	7 (46.6%)	0.153
Non-progress of labour	3 (12.5%)	1 (6.6%)	1.0
Intrapartum bleeding	1 (4.16%)	0	1.0
Maternal nasal O_2 requirement	6 (20%)	0	**0.024**
Neonatal PCR positive tests	0 (0%)		
Gestational diabetes mellitus	1 (3.3%)	0	0.492
NICU requirement	7 (23.3%)	2 (6.7%)	0.145
Maternal Blood Pressure			
SP (mmHg)	121.0±7.8	123.0±9.8	0.385
DP (mmHg)	81.0±4.8	83.0±5.2	0.127
Insertion anomaly	0 (0%)	3 (10.0%)	0.237
Maternal-obstetric complications n (%)	4 (13.3%)	1 (3.3%)	0.353
Uterine atony	1 (3.3%)	0	
Oligohydramnios	1 (3.3%)	1 (3.3%)	
Polyhydramnios	1 (3.3%)	0	
Premature rupture of membranes	1 (3.3%)	0	

*Independent t-test, Mann-Whitney U test and Fisher's Exact test, where appropriate. Data are given as mean ± SD, n (%). BMI: Body mass index, CRP: C-reactive protein, WBC: White blood cell, C/S: Cesarean/section, NICU: Neonatal intensive care unit, SP: Systolic pressure, DP: Dyastolic pressure, COVID: Coronaviruse disease, PCR: Polymerase chain reaction

suggested that even though SARS-CoV-2 could not be examined with PCR in the placental tissues, considering PCR positivity in the newborns, the maternal immune response in the placenta of asymptomatic women was of secondary vascular changes similar to that of symptomatic patients.

The correlation between placental findings and their impact on clinical features is a controversial issue. According to Linehan et al.[26] perivillous fibrinoid accumulation and necrotic trophoblast remnants were described as placental findings in pregnant women with COVID-19, however reported that no complications developed in either the mothers or the newborns. In another study, Chen et al.[27] identified massive infarction, diffuse fibrinoid deposition, and local increases in syncytial nodes in the placental pathologies of pregnant women with COVID-19, but no adverse events were seen in the mothers and infants either during pregnancy or postpartum. Hsu et al.[28] reported chronic villitis, hypertrophic arteriolopathy and extravillous trophoblast islands in the placentas of COVID-19-positive patients, but these findings had no negative clinical effects on mother or infant. These studies showing that the placental vascular structure was affected were conducted on patient populations with predominant clinical COVID-19 symptoms, and this raises the question of the relationship between disease severity and the effect on the placenta in asymptomatic cases. The impact of COVID-19 on maternal and fetal health remains a topic of research interest. Tasca et al.[11] compared pregnant women with COVID-19 positivity with healthy pregnant women and suggested that COVID-19 does

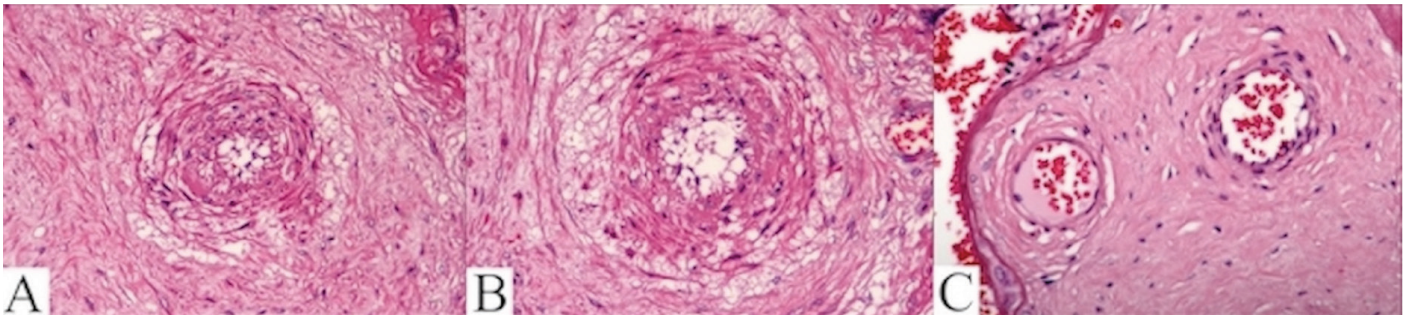

Figure 1. A-B: Mural hypertrophy of the vessel wall in placentas of COVID pregnancies. HE x400. C: Normal small sized vessels of placenta from a healthy control. HE x400

Table 3. Comparison of pathological findings between groups

	COVID (+) n=30	COVID (-) n=30	p-value*
Cord vascular ectasia	13 (43.3%)	8 (26.7%)	0.176
Cord vascular thrombosis	2 (6.7%)	2 (6.7%)	1.000
Placental infarcts	14 (46.7%)	10 (33.3%)	0.292
Retroplacental hemorrhage	2 (6.7%)	0 (0%)	0.150
Chorangiosis	3 (10.0%)	8 (26.7%)	0.095
Distal villous hypoplasia	19 (63.3%)	12 (40.0%)	0.071
Accelerated villous maturation	1 (3.3%)	5 (16.7%)	0.085
Decidual arteriopathy	1 (3.3%)	2 (6.7%)	1.000
Thrombosis	2 (6.7%)	1 (3.3%)	1.000
Avascular villi	12 (40.0%)	8 (26.7%)	0.273
Villous stromal karyorrhexis	0	0	
Mural hypertrophy	25 (83.3%)	9 (30.0%)	**0.001**
Stem villous obliteration	6 (20.0%)	5 (16.7%)	0.739
Delayed villous maturation	0	0	
Funisitis	0	0	
Chorioamnionitis	1 (3.3%)	2 (6.7%)	1.000
Villitis	0	0	
Perivillous fibrin deposition	17 (56.7%)	23 (76.7%)	0.100

All data are given as n (%). *Fisher's Exact test, COVID: Coronavirus disease

not have unfavorable effects in terms of delivery mode, placental weight, newborn weight and 5 min. APGAR scores. Similarly, Jaiswal et al.[15] suggested that the presence of COVID-19 did not negatively influence maternal complications, except fever, which may be the result of COVID-19. In this study, no significant differences were determined regarding maternal-obstetric complications and fetal-neonatal parameters including birthweight, NICU admissions, and 5 min. APGAR scores, which were similar in both groups, despite the higher rate of placental abnormalities in the COVID-19-positive group. The lack of association between these underlying conditions in this study may be due to secondary. Type II error resulting from a small sample size, and therefore, further studies with larger sample sizes may help identify associations. Controversially, these results may suggest that the placenta acts as a protective biological filter, or actively produce factors that may protect the fetus in utero. Furthermore, in mothers infected with SARS-CoV-2, the fetus may also be protected by vertical transmission of immunoglobulins from the mother to the fetus through the blood supply[29]. However, the current study findings suggest that damage to the placenta develops with possible adverse effects on the neonates, warranting follow-up for possible side effects.

The rate of delivery by cesarean section was significantly higher in COVID-19-positive pregnant women, which may be related to "maternal desire associated with reduced contact with the patient and want a more organized intervention". However, there was a significant increase in the need for maternal nasal O_2 support in asymptomatic cases. Similar to previous studies[8], this may have been related to an increase in the oxygen requirement during vaginal or cesarean delivery.

As a secondary objective, a significant difference was observed in lymphopenia and CRP and ferritin elevation when asymptomatic COVID-19 patients were compared with control subjects. Although these findings are consistent with the literature[30,31], they show a correlation with the severity of the disease and suggest that there is a change in laboratory parameters before the development of symptoms in asymptomatic cases. Additionally, no relationship was observed between mural hypertrophy and laboratory parameters in this study. As there are no studies in the literature that have investigated the relationship between placental pathologies and laboratory parameters, there is an obviously need for more research on this subject.

In the study, which included asymptomatic COVID-19-positive pregnant women, mural hypertrophy was a striking finding with respect to placental VM findings. The determination of VM in the placenta has been associated especially with the comorbidities of hypertension and preeclampsia in pregnancy[32]. However, as these comorbidities were excluded

Table 4. Factors affecting the formation of mural hypertrophy in vessel walls

	Mural Hypertrophy		Univariate		Multivariate	
	Negative (n=26)	Positive (n=34)	OR (95% CI)	p-value	OR (95% CI)	p-value
Age	27.73±6.53	29.65±5.43	1.058 (0.968 - 1.156)	0.217	0.996 (0.872 - 1.137)	0.950
Pregnancy week	38.32±1.69	38.11±1.71	0.927 (0.683 -1.260)	0.630	1.083 (0.700 - 1.675)	0.720
BMI	24.52±2.59	23.6±2.92	0.886 (0.733 - 1.070)	0.209	0.806 (0.613 - 1.061)	0.124
CRP	7.92±11.08	32.77±36	1.066 (1.013 - 1.122)	**0.014**	1.032 (0.964 - 1.104)	0.365
Ferritin	46.06±27.64	235.54±364.16	1.011 (0.999 - 1.023)	0.065	1.001 (0.992 - 1.011)	0.775
WBC	10.72±3.87	10.15±3.42	0.956 (0.828 - 1.104)	0.540	0.940 (0.731 - 1.208)	0.630
NLR	4.41±2.69	5.62±2.65	1.209 (0.966 - 1.513)	0.097	1.241 (0.906 - 1.699)	0.179
Type of delivery						
Normal	12 (57.1)	9 (42.9)	Reference		Reference	
Cesarean	14 (35.9)	25 (64.1)	2.381 (0.805 - 7.039)	0.117	1.612 (0.302 - 8.596)	0.576
Complications						
Negative	20 (40.8)	29 (59.2)	Reference		Reference	
Positive	6 (54.5)	5 (45.5)	0.575 (0.154 - 2.144)	0.410	0.303 (0.046 - 2.021)	0.218
COVID-19						
Negative	21 (70)	9 (30)	Reference		Reference	
Positive	5 (16.7)	25 (83.3)	11.667 (3.384 - 40.22)	**<0.001**	4.716 (1.012 - 22.251)	**0.048**

Data are given as n (%) or mean ± SD. OR: Odds ratio, CI: Confidince interval, BMI: Body mass index, CRP: C-reactive protein, WBC: White blood cells, NLR: Neutrophil to lymphocyte ratio, COVID-19: Coronavirus disease, SD: Standard deviation

in this study, this suggests that the finding of VM could be specific to COVID-19[33]. Since there was no difference between the groups in terms of comorbidities during pregnancy and neonatal follow-up, it was thought that the presence of placental vascular pathology could have been related to a placental effect even in asymptomatic cases and perhaps not enough time had passed for negative outcomes to have formed.

Strong aspects of this study can be considered to be that it is the first prospective case-control study to review the placental findings at the time of delivery of asymptomatic COVID-19-positive pregnant women. Additionally, hypertensive patients were excluded as that could have caused placental vascular pathologies, and asymptomatic COVID-19 patients were included, which is the most common but least studied patient population[33].

Study Limitations

However, the study also had some limitations; the number of patients was relatively low, although it was within acceptable limits for the power of the study. A second limitation was the focus on only the effects of COVID-19 during pregnancy and the neonatal period, and the lack of short-term and long-term results. Thirdly, this study examined the experience of a single centre, and therefore, further studies including data from more than one academic centre will undoubtedly make a significant contribution to explaining the impact of COVID-19 on pregnancy and the placenta. A final limitation could be considered to be that the sensitivity of the antibody tests used in the selection of the case and control groups has been reported to be 50-70%[34].

Conclusion

This study demonstrated that asymptomatic COVID-19 positivity in the perinatal period had no significant effect on the pregnancy or neonatal complications. However, mural hypertrophy in the placenta was detected at a significantly high rate in the COVID-19 patients. Although concerns about placental vasculopathy increase in COVID-19-positive pregnant women, further studies may clarify the importance of histological placental findings on maternal and neonatal health.

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Ethics

Ethics Committee Approval: Ethics committee approval was obtained from the local "Medical Ethics and Institutional Review Board" with ID number E-48670771-514.10/136 in April 19, 2021, and the study was completed in accordance with Helsinki Declaration principles.

Informed Consent: All the pregnant women provided informed consent to participate in the study.

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Authorship Contributions

Surgical and Medical Practices: O.Ş., A.Y.A., E.A., H.B., Ö.Y., Concept: O.Ş., A.Y.A., E.A., H.B., Ö.Y., Design: O.Ş., A.Y.A., E.A., H.B., Ö.Y., Data Collection or Processing: O.Ş., A.Y.A., E.A., H.B., Ö.Y., Analysis or Interpretation: O.Ş., A.Y.A., E.A., H.B., Ö.Y., Literature Search: O.Ş., A.Y.A., E.A., H.B., Ö.Y., Writing: O.Ş., A.Y.A., E.A., H.B., Ö.Y.

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The predictive role of second trimester uterocervical angle measurement in obstetric outcomes

İkinci trimester uteroservikal açı ölçümünün obstetrik sonuçları öngörmedeki rolü

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Abstract

Objective: Uterocervical angle has been suggested as a marker to predict preterm birth. However, the literature has limited data about its predictive role in preterm delivery. Moreover, no evidence is present to clarify the role of second-trimester uterocervical angle in induction success and postpartum hemorrhage. Here, it was aimed to compare the role of uterocervical angle with cervical length in predicting preterm labor and assess the utility of the second-trimester uterocervical angle in induction success and postpartum hemorrhage.

Materials and Methods: A total of 125 pregnant women, hospitalized with a diagnosis of preterm labor were included in the study. Sonographic measurements of cervical length and uterocervical angle were performed between 16 and 24 weeks of gestation. The demographic, obstetric, laboratory, and sonographic features of the participants were recorded. Patients were divided into subgroups as preterm and term; with and without induction success; with and without postpartum hemorrhage. Additionally, preterm cases were divided into subgroups as early and late preterm. Variables were evaluated between the groups.

Results: Cervical length was shorter in the preterm group (30.74±6.37 and 39.19±5.36, p<0.001). The uterocervical angle was 100.85 (85.2-147) in preterm and 88 (70-131) degrees in terms that were statistically significant (p<0.001). Furthermore, the uterocervical angle was wider [126 (100.7-147) and 98 (85.2-114), p<0.001] in the early preterm group. When the groups with and without postpartum bleeding were compared, no significant difference was detected in terms of uterocervical angle [96.5 (71-131) and 88 (70-147), p=0.164]. Additionally, the uterocervical angle was wider in the successful induction group (p<0.001). An a uterocervical angle >85 degrees predicted preterm delivery with 100% sensitivity and 45.54% specificity [area under the curve (AUC)=0.743, p<0.001]. When the cervical length and uterocervical angle were evaluated together to predict preterm delivery, no significant difference was found (p=0.086). An a uterocervical angle >88 degrees predicted induction success with 84.78% sensitivity and 79.75% specificity (AUC=0.887, p<0.001).

Conclusion: Our study revealed that the uterocervical angle can be a useful marker in predicting preterm labor and induction success, although it does not predict postpartum hemorrhage.

Keywords: Induction success, postpartum hemorrhage, preterm labor, cervical length, uterocervical angle

Öz

Amaç: Bu çalışmada uteroservikal açının preterm eylem ve obstetrik sonuçları öngörmedeki rolünü değerlendirmeyi amaçladık.

Gereç ve Yöntemler: On altı-24 hafta arası rutin takip için polikliniğe başvuran, uteroservikal açısı ölçülerek doğumu tarafımızca yaptırılan toplam 125 gebeyi çalışmaya dahil ettik. Hastaların yaş, boy, kilo, gravida, parite, doğum şekli, gebelik haftası ve fetal ağırlık, doğum öncesi ve doğum sonrası tam kan sayımı, servikal uzunluk ve uteroservikal açı değerleri kaydedildi. Hastalar preterm ve term; indüksiyon başarısı olan ve olmayan; doğum sonu kanama olan ve olmayan olarak ayrıldı. Değişkenler gruplar arasında değerlendirildi.

PRECIS: Second trimester uterocervical angle can be a useful marker in predicting preterm labor and induction success, while it does not predict postpartum hemorrhage.

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Bulgular: Servikal uzunluk preterm grupta anlamlı olarak daha kısaydı (30,74±6,37 ve 39,19±5,36, p<0,001). Uteroservikal açı pretermlerde 100,85 (85,2-147) ve termlerde 88 (70-131) derece idi ve istatistiksel olarak anlamlıydı (p<0,001). Ayrıca erken preterm grubunda uteroservikal açı anlamlı olarak daha genişti [126 (100,7-147) ve 98 (85,2-114), p<0,001]. Doğum sonu kanaması olan ve olmayan gruplar karşılaştırıldığında uteroservikal açı [96,5 (71-131) ve 88 (70-147) açısından anlamlı fark bulunmadı, p=0,164]. Hastalar indüksiyon başarısına göre sınıflandırıldığında, indüksiyon başarısı pozitif grupta uteroservikal açı daha genişti (p<0,001). Uteroservikal açı >85 derece olması, preterm doğumu %100 duyarlılık ve %45,54 özgüllük ile öngördü (eğrinin altındaki alan=0,743, p<0,001).

Sonuç: Çalışmamız literatürle uyumlu olarak uteroservikal açının preterm eylem ve indüksiyon başarısını öngörmede yararlı bir belirteç olabileceğini gösterdi. İlaveten bu açı, erken ve geç preterm olgularda da yararlı bir belirteç olabilir.

Anahtar Kelimeler: İndüksiyon başarısı, postpartum kanama, preterm eylem, servikal uzunluk, uteroservikal açı

Introduction

Preterm labor, which is defined as births before 37 weeks of gestation, is one of the most common obstetric complications worldwide. Although the pathogenesis of preterm labor is not clearly understood, intraamniotic infection or bleeding, uteroplacental ischemia, overdistension of the uterus, and immunological processes are proposed in the etiology. The prediction of preterm labor plays a crucial role in avoiding premature births and related complications. However, there is still no precise predictive tool[1]. Many obstetricians have proposed different ultrasonographic measurements and biochemical markers to predict true preterm labor. Sonographic assessment of cervical structure by measuring cervical length (CL) has been used as a popular predictive tool to predict preterm labor. The uterocervical angle (UCA) is defined as the angle between the lower anterior uterine segment and the endocervical canal. Recently, UCA has been suggested as an alternative to CL to predict preterm birth. Additionally, the UCA is supposed to play a predictive role in induction success, primary dysmenorrhea, cerclage failure, unexplained infertility, and second-trimester pregnancy terminations[2-4]. Unfortunately, the data about the relationship between wider UCA and induction success is not clear[5,6]. To the best of our knowledge, there is no study investigating the role of the second-trimester UCA in induction success and postpartum hemorrhage.

This study compared the predictive role of UCA with CL in predicting preterm labor and assess the utility of the second trimester UCA in induction success and postpartum hemorrhage.

Materials and Methods

A single-center, prospective study was conducted in a university-affiliated research and training hospital between December 1, 2020, and June 30, 2021.

A total of 125 pregnant women, hospitalized with a diagnosis of preterm labor were included in the study. This study was approved by the local ethics committee with an approval number 2011-KAEK-25 and complied with the Helsinki Declaration. Written informed consent was obtained from all participants.

Preterm labor was defined as births that occurred before 37 weeks of gestation. Then, it was classified as early preterm (before 34 weeks) or late preterm (between 34 and 36 weeks). Inclusion criteria for the study were as follows: i) singleton pregnant women between 18 and 40 years old, ii) being in the second trimester of the pregnancy and having a fetus in a vertex presentation, iii) having a sonographic measurement of CL and UCA, iv) giving birth in the hospital. Pregnant women younger than 18 years old, having chronic diseases, uterine anomalies, previous uterine surgery, multiple pregnancies, and cigarette smokers, alcohol consumers were excluded. Additionally, pregnant women having a history of preterm delivery or postpartum hemorrhage were excluded from the study. Patients with a moderate or high risk of postpartum hemorrhage were also excluded from the study.

CL and UCA measurements were conducted between 16 and 24 gestational weeks by the same physician (MS). After the bladder of the patients had been emptied, transvaginal sonography was performed in the lithotomy position. The vaginal probe of the Voluson P6 Model Ultrasonography device was placed into the vagina without pressing on the cervix. UCA was defined as the angle between the anterior uterine segment and the internal cervical os. Then, the distance between the internal os and the external cervical os was recorded as CL. While defining CL, a cross-section image was taken in the sagittal plane, from where the internal cervical os, external cervical os, cervical canal, and endocervical mucosa can be viewed simultaneously and occupied 3/4 of the screen. If the two os were located on a single line, the distance between the two was measured directly. If it is not on the same line, the linear parts were measured separately and summed up to obtain the CL. Each measurement for CL and UCA was performed three times and then mean values were calculated in the analysis.

The vaginal ovule of dinoprostone was applied to all participants with a bishop score of ≤6. The maximum application duration of the dinoprostone ovule was 24 h[7]. The time of application was recorded and patients with no cervical dilatation despite dinoprostone for 24 h, were performed a cesarean section and excluded from the study. While the Bishop score was more than 6, the dinoprostone oval was removed and oxytocin was infused. It was prepared as 5 units in 500 mL saline and was started with an initial dose of 4 mU/min which was increased 2 mU/min every 20 min. The maximum dose was defined as 20 mU/min. Continuous fetal heart monitoring was performed for all patients having uterine contractions.

Postpartum hemorrhage was defined as an estimated blood loss above 1000 mL. Estimated blood loss was calculated as estimated blood volume x (preoperative hematocrit - postoperative hematocrit)/preoperative hematocrit [where estimated blood volume (mL) = weight (Kg) x 85][8].

The demographic and obstetric characteristics of the patients, such as age, body mass index [weight (kg)/height x height (m^2)], gravida, parity, delivery week, delivery mode, and birth weight were recorded. Additionally, laboratory and sonographic characteristics such as prepartum and postpartum hemoglobin, hematocrit, white blood cell (WBC) and sonography week, CL, and UCA values were also recorded.

Statistical Analysis

Statistical analysis was carried out using SPSS Version 23.0. (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Armonk, NY: IBM Corp.) and Medcalc version 19.5.6 software. Shapiro-Wilk test was performed to evaluate the normality of the distribution of the variables. The Student t-test was used to compare normally distributed continuous variables, while the Mann-Whitney U test was used to compare non-normally distributed continuous variables between two groups. For categorical variables, chi-square and Fisher's exact tests were used for comparisons. The descriptive statistics were expressed as mean ± standard deviation for normally distributed variables, median (minimum-maximum) for non-normally distributed variables, and frequency or percentages for categorical variables. Receiver operating curve (ROC) analysis was carried out to examine the efficiency and cut-off values of UCA in predicting preterm delivery and induction success. An alfa value <0.05 was considered statistically significant.

Results

A total of 125 pregnant women were included in the study and participants were divided into two groups: Preterm (n=24) and term (n=101). The demographic, obstetric, laboratory, and sonographic characteristics of the two groups are shown in Table 1. No statistically significant difference was determined between the two groups in terms of age, gravida, parity, body mass index, delivery mode, postpartum hemorrhage, sonography week, prepartum and postpartum hemoglobin, hematocrit, and WBC values. As expected, delivery week and birth weight was significantly lower in the preterm group. The mean of the CL in the preterm and term groups was 30.74±6.37 mm and 39.19±5.36 mm, respectively. A statistically significant difference was obtained between the two groups (p<0.001). The UCA was 100.85 (85.2-147) degrees in the preterm group and

Table 1. Demographic, obstetric, laboratory and sonographic characteristics of term and preterm groups

	Preterm (n=24)	Term (n=101)	p
Age (years)	27.5 (19-35)	26 (18-37)	0.121
Gravida (n)	2 (1-8)	2 (1-8)	0.370
Parity (n)	1 (0-4)	1 (0-4)	0.487
BMI (kg/m^2)	27.78 (16.53-40.86)	25.46 (19.13-38.67)	0.311
Delivery week (week)	36 (25-36)	38 (37-41)	**<0.001**
Delivery mode			
- Vaginal	22 (91.7%)	57 (56.4 %)	0.003
- Cesarean section	2 (8.3%)	44 (43.6 %)	
Birth weight (gram)	2.885 (650-3600)	3.200 (2435-4210)	**<0.001**
Postpartum hemorrhage (n, %)	3 (12.5%)	21 (20.8%)	0.564
Prepartum hemoglobin (g/dL)	11.42±1.13	11.7±1.14	0.275
Postpartum hemoglobin (g/dL)	10.7±1.41	10.77±1.22	0.795
Prepartum hematocrit	35 (25.1-40)	34.9 (21.2-42.8)	0.670
Postpartum hematocrit	32.25 (19-38.9)	32.2 (24.1-42.1)	0.948
Prepartum WBC (mcL)	10.900 (6.700-22.400)	10.600 (6.700-16.000)	0.564
Postpartum WBC (mcL)	15.050 (7.200-25.000)	14.800 (8.500-28.000)	0.656
Sonography time (week)	20 (16-23)	20 (16-24)	0.820
Cervical length (mm)	30.74±6.37	39.19±5.36	**<0.001**
Uterocervical angle (degree)	100.85 (85.2-147)	88 (70-131)	**<0.001**

Values were presented as mean ± SD or n (%) or median (min-max). P-value <0.05 was statistically significant. SD: Standard deviation, BMI: Body mass index, WBC: White blood cell, Min: Minimum, Max: Maximum

88 (70-131) degrees in the term group. A statistically significant difference was noticed between the two groups in terms of UCA (p<0.001). The role of CL and UCA in predicting preterm delivery was evaluated thanks to ROC analysis. CL value of ≤33 millimeters had sensitivity of 70.83% and specificity of 86.14% [area under the curve (AUC)=0.847, p<0.001] (Figure 1a), and a UCA value of more than 85 degrees had a sensitivity of 100% and a specificity of 45.54% (Figure 1b). Moreover, when the cut-off value was taken to 95, as it is in the literature, UCA had a sensitivity of 70.83% and specificity of 63.37%. When the role of CL and UCA in predicting preterm delivery was evaluated together, no statistically significant difference was detected between the two AUC values (p=0.086) (Figure 1c).

Preterm cases were classified as early (n=7) or late (n=17). The demographic, obstetric, laboratory, and sonographic characteristics of the two groups are shown in Table 2. No statistically significant difference was detected between these groups according to age, gravida, parity, body mass index, sonography week, prepartum and postpartum hemoglobin, hematocrit, and WBC. Delivery week and birth weight was significantly lower in the early preterm group (p<0.001 and p=0.011, respectively). The CL was significantly shorter [25 (15.5-30) mm vs 32 (23-42.5) mm, p<0.001], and UCA was significantly narrower [126 (100.7-147) degrees vs 98 (85.2-114) degrees, p<0.001] in the early preterm group.

When postpartum hemorrhage cases based on hemoglobin decrease were diagnosed evaluated, 24 cases were diagnosed with postpartum hemorrhage. The demographic, obstetric, laboratory, and sonographic characteristics of the postpartum hemorrhage and control groups are presented in Table 3. No significant difference was detected between the two groups with regard to age, gravida, parity, body mass index, delivery week and mode, birth weight, sonography week, prepartum hematocrit, prepartum and postpartum WBC. Furthermore, CL (37.98±5.06 vs 37.48±6.78, p=0.734) and UCA [96.5 (71-131) vs 88 (70-147), p=0.164] were not significantly different between the two groups.

Patients were also divided into two successful subgroups (n=79) and unsuccessful (n=46) induction groups. Demographic, obstetric, laboratory, and sonographic characteristics of the groups are shown in Table 4. No significant difference was found in terms of age, gravida, parity, body mass index, delivery week, birth weight, prepartum and postpartum hemoglobin, hematocrit, WBC, sonography week, and CL. UCA was significantly wider in the successful induction group compared to the unsuccessful induction group [(73.4-147) vs. 78.5 (70-110), p<0.001]. In ROC analysis (Figure 2), UCA was calculated to predict induction success with a cut-off value of 88 degrees, sensitivity of 84.78%, and a specificity of 79.75% (AUC=0.887, p<0.001).

Discussion

The main findings of this study revealed that the second-trimester UCA value played a predictive role for preterm birth and no difference was present between UCA and CL in this prediction. Additionally, anterior UCA measured in the second trimester was found to predict the success of labor induction. However, UCA played no predictive role in postpartum hemorrhage.

UCA, which presents the angle between the anterior uterine wall and cervical canal, is a newly used ultrasonographic parameter for predicting many obstetric outcomes. In the literature, there are studies investigating the role of UCA in preterm birth, labor induction, pregnancy termination, the success of cerclage, dysmenorrhea, polyhydramnios, and unexplained infertility[2,3,6,9,10]. Narrow UCA is related to unexplained

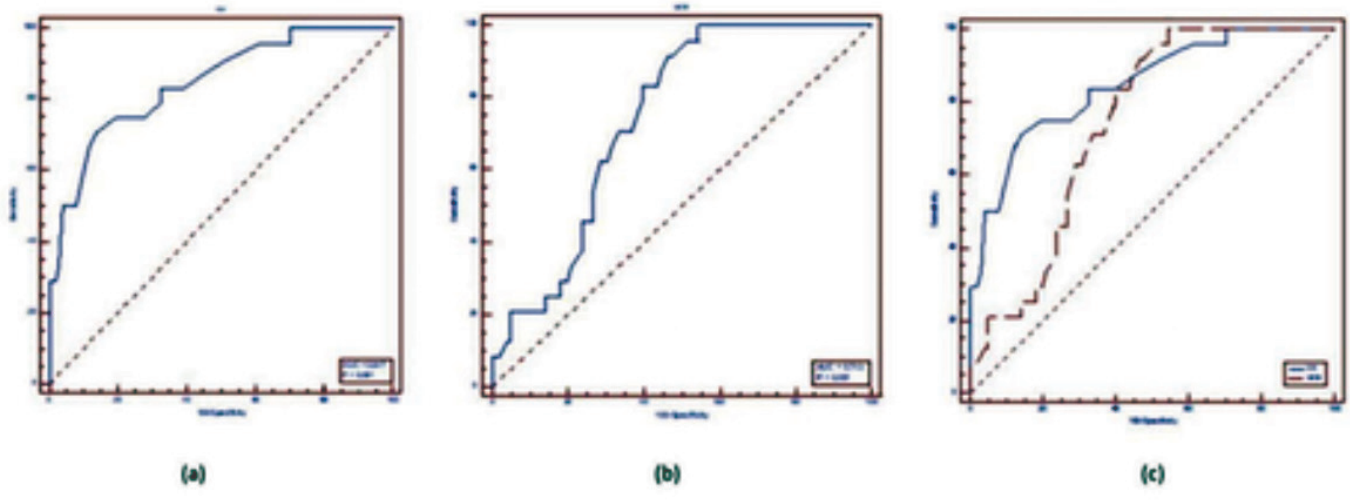

Figure 1. The ROC analysis to determine the predictive role of (a) cervical length, (b) uterocervical angle, (c) cervical length and uterocervical angle

ROC: Receiver operating curve

infertility[10] and primary dysmenorrhea[11], whereas broader UCA values are associated with successful second-trimester termination and preterm labor[12,13].

Recent studies have shown that broader UCA measured in the second trimester is linked to spontaneous preterm birth in singleton pregnancies[9,14]. In 2016, Dziadosz et al.[15] measured CL and UCA in 972 pregnant women between 16 and 24 weeks of gestation. In this study, the UCA of >95 degrees was found to predict preterm birth <37 weeks with a sensitivity of 80%, and the UCA of >105 degrees predict preterm birth <34 weeks with sensitivity of 81%. Moreover, it was claimed that CL ≤25 mm predicts preterm birth at both <34 weeks and <37 weeks nearly with a sensitivity of 62%. In the study of Sawaddisan et al.[16], it was shown that UCA was significantly wider in patients with spontaneous birth compared with term births. Furthermore, a UCA of ≥110 degrees predicted preterm birth with a sensitivity of 83.3% and specificity of 61.2%. In another study[17], Luechathananon evaluated the role of UCA and CL in predicting preterm birth in threatened preterm delivery and reported that the UCA of ≥110.97 degrees predict preterm birth with 65.1% sensitivity and 43.6% specificity, the CL of <34 mm predict preterm birth with 48.8% sensitivity and 68.4% specificity. In contrast, Farràs Llobet et al.[18] claimed that the UCA measured between 19 and 23 gestational weeks in singleton pregnancies had been a poor predictor of spontaneous preterm birth. Likewise, Wagner et al.[19] evaluated this role in women with pain, who have regular uterine contractions and a CL of ≤25 mm and reported that the UCA had not been a useful predictor of preterm birth after one week since the initiation of preterm contractions.

In this study, it was concluded that the second trimester UCA was wider in the preterm birth group, and the UCA of >85 degrees had a sensitivity of 100% and specificity of 45.54% for predicting preterm birth. Moreover, when the cut-off value

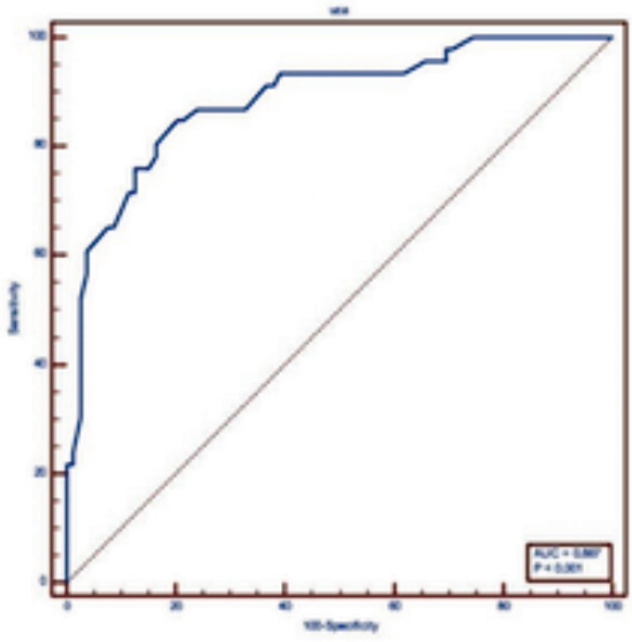

Figure 2. The ROC analysis to determine the predictive role of uterocervical angle in induction success

ROC: Receiver operating curve

Table 2. Demographic, obstetric, laboratory and sonographic characteristics of early and late preterm groups

	Early preterm (n=7)	Late preterm (n=17)	p
Age (years)	32 (23-35)	27 (19-35)	0.065
Gravida (n)	2 (1-3)	2 (1-8)	0.804
Parity (n)	1 (0-2)	1 (0-4)	0.951
BMI (kg/m²)	30.3 (17-36.44)	27.55 (16.53-40.86)	0.288
Delivery week (week)	30 (25-34)	36 (35-36)	**<0.001**
Birth weight (gram)	1.200 (650-3034)	2.980 (1640-3600)	**0.011**
Sonography time (week)	20 (16-23)	21 (16-23)	0.757
Prepartum hemoglobin (g/dL)	11.2 (8.6-13.5)	11.7 (10-12.9)	0.951
Postpartum hemoglobin (g/dL)	11 (8.7-13.7)	10.4 (7.9-12.6)	0.418
Prepartum hematocrit	34.7 (25.1-38.6)	35 (30-40)	0.534
Postpartum hematocrit	35.6 (26-37.3)	32 (19-38.9)	0.383
Prepartum WBC (mcL)	10.600 (8.300-20.000)	11.200 (6.000-22.400)	0.710
Postpartum WBC (mcL)	15.800 (7.200-22.800)	14.900 (10.300-25.000)	0.576
Cervical length (mm)	25 (15.5-30)	32 (23-42.5)	**<0.001**
Uterocervical angle (degree)	126 (100.7-147)	98 (85.2-114)	**<0.001**

Values were presented as median (min-max). P-value <0.05 was statistically significant. BMI: Body mass index; WBC: White blood cell, Min: Minimum, Max: Maximum

Table 3. Demographic, obstetric, laboratory and sonographic characteristics of postpartum hemorrhage and control groups

	Postpartum hemorrhage (n=24)	Control (n=101)	p
Age (years)	27.5 (18-35)	26 (18-37)	0.585
Gravida (n)	2.5 (1-5)	2 (1-8)	0.612
Parity (n)	1 (0-3)	1 (0-4)	0.460
BMI (kg/m²)	28.85 (22.06-34.45)	25.7 (16.53-40.86)	0.705
Delivery week (week)	38 (25-40)	38 (26-41)	0.796
Delivery mode			
- Vaginal	18 (75%)	61 (60.4%)	0.272
- Cesarean section	6 (25%)	40 (39.6%)	
Birth weight (gram)	3.145 (750-3820)	3.105 (650-4210)	0.590
Hemoglobin change (%)	-18.18 (-30:-15)	-5.5 (-18:17)	**<0.001**
Prepartum hematocrit	34.89±4.88	34.47±3.24	0.604
Hematokrit change (%)	-12 (-47:74)	-5 (-28:17)	**<0.001**
Prepartum WBC (mcL)	11.250 (8.000-22.400)	11.500 (6.700-20.000)	0.225
Postpartum WBC (mcL)	15.250 (10.500-25.000)	14.600 (7.200-28.000)	0.263
Sonography time (week)	20 (16-24)	20 (16-24)	0.980
Cervical length (mm)	37.98±5.06	37.48±6.78	0.734
Uterocervical angle (degree)	96.5 (71-131)	88 (70-147)	0.164

Values were presented as mean ± SD or n (%) or median (min-max). P-value <0.05 was statistically significant. SD: Standard deviation, BMI: Body mass index, WBC: White blood cell, Min: Minimum, Max: Maximum

Table 4. Demographic, obstetric, laboratory and sonographic characteristics of successful and unsuccessful induction groups

	Successful induction (n=79)	Unsuccessful induction (n=46)	p
Age (years)	27.43±4.44	26±5.29	0.109
Gravida (n)	2 (1-8)	2 (1-8)	0.622
Parity (n)	1 (0-4)	1(0-4)	0.550
BMI (kg/m²)	27.7±5.08	25.19±3.48	0.016
Delivery week (week)	38 (225-41)	38 (36-41)	0.017
Birth weight (gram)	3.100 (6504040)	3.200 (2460-4210)	0.274
Prepartum hemoglobin (g/dL)	11.56±1.11	11.78±1.19	0.292
Postpartum hemoglobin (g/dL)	10.58±1.14	11.07±1.38	0.053
Prepartum hematocrit	35 (21.2-42.8)	34.95 (24-40.9)	0.441
Postpartum hematocrit	32 (19-40.2)	33 (24.1-42.1)	0.354
Prepartum WBC (mcL)	10.800 (6.700-22.400)	10.600 (6.700-16.000)	0.693
Postpartum WBC (mcL)	14.700 (7.200-25.000)	150.020 (9.000-28.000)	0.229
Sonography time (week)	20 (16-24)	20 (16-24)	0.572
Cervical length (mm)	36.97±6.47	38.61±6.41	0.173
Uterocervical angle (degree)	100 (73.4-147)	78.5 (70-110)	**<0.001**

Values were presented as mean ± SD or median (min-max). P-value <0.05 was statistically significant. SD: Standard deviation, BMI: Body mass index, WBC: White blood cell

was taken to 95 degrees, as in the literature, the UCA had a sensitivity of 70.83% and specificity of 63.37%. Additionally, the CL of ≤33 millimeters had a sensitivity of 70.83% and specificity of 86.14% in the prediction of preterm birth. When the predictive roles of CL and UCA were compared, no differences were found. The study was performed in a low-risk population for preterm birth and the findings were consistent with the literature. Another interesting finding of the study was the significant difference in UCA values between early and late preterm cases. Unfortunately, ROC analysis could not be performed because to the limited number of cases.

In 2018, Eser and Ozkaya[6] searched the role of UCA in labor induction and suggested that the CL and UCA could be predictors of successful labor induction. Optimal cut-off values were reported as 97 degrees with 64% sensitivity and 91% specificity for UCA and 27 mm with 64% sensitivity and 64% specificity for CL. In another study by Dagdeviren et al.[5], the predictive effect of UCA on labor induction was explored and concluded that the pre-induction UCA could not predict labor induction, whereas the broader UCA led to shortened active phase duration.

In this study, it has been the first time to investigate the role of second-trimester UCA instead of pre-induction in the prediction of induction success. It was concluded that the second-trimester UCA was wider in the successful induction group and it predicted induction success with a cut-off value of 88 degrees, with a sensitivity of 84.78% and specificity of 79.75%. In addition to the literature, the role of the second trimester UCA was evaluated in postpartum hemorrhage and no significant difference was found between postpartum hemorrhage and control groups.

Study Limitations

However, this study had some limitations. First, the number of patients who participated in the study was limited. It was thought that more study participants would yield more robust results. Second, the UCA was measured between 16 and 24 weeks of gestation and the measurement week was not considered. Only Sawaddisan et al.[20] evaluated the UCA prediction success according to the weeks and reported a significant difference between the groups when the UCA measurement was taken only over 19.5 weeks.

Conclusion

Consequently, the second-trimester UCA was found to be a useful predictive marker for preterm birth in low-risk, singleton pregnancies and had a significant difference between early and late preterm cases. Furthermore, the second-trimester UCA can predict induction success, while it has no use in postpartum hemorrhage. New studies are needed to increase the diagnostic accuracy of the UCA in preterm labor with more specific patient groups and cut-off values that can be standardized. Additionally, further studies with much more participants should be conducted to confirm these findings.

Ethics

Ethics Committee Approval: This study was approved by the local ethics committee with an approval number 2011-KAEK-25 and complied with the Helsinki Declaration.

Informed Consent: Written informed consent was obtained from all participants.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: M.Ş., E.Ü., B.D.Ç., S.K., N.N.Y., Concept: M.Ş., E.Ü., B.D.Ç., S.K., N.N.Y., Design: E.Ü., B.D.Ç., S.K., Data Collection or Processing: M.Ş., S.K., Analysis or Interpretation: B.D.Ç., N.N.Y., Literature Search: B.D.Ç., S.K., N.N.Y., Writing: M.Ş., E.Ü., B.D.Ç., S.K., N.N.Y.

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Comparison of the localization of intrauterine adhesions in pregnant and infertile women

Gebe ve infertil kadınlarda intrauterin adezyonların lokalizasyonlarının karşılaştırılması

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Abstract

Objective: Intrauterine adhesion (IUA) is the formation of band-shaped fibrotic tissues in the endometrial cavity due to uterine procedures. Most adhesions remain asymptomatic and do not affect fertility or pregnancy conditions. However, they may lead to infertility and pregnancy complications in some women. This study aimed to determine which localization and type of IUA may lead to infertility.

Materials and Methods: Seventy-six women with IUA were retrospectively scanned. Thirty-nine women with IUA with uterine factor-related infertility were included in the infertility group. Thirty-seven pregnant women, who had adhesions in the second-trimester ultrasonography and who had a live birth via cesarean section at term, were included in the pregnancy group. The localization of adhesions was determined as the fundus, corpus, isthmus, and cornu. Concerning the type of adhesion, the adhesions were classified as dense- and film-type adhesions.

Results: The infertility group was compared with the pregnancy group according to the type and localization of the adhesions. Fundal adhesions were significantly higher in the infertility group compared to the pregnancy group ($p<0.05$). The isthmic adhesions, however, were more common in the pregnancy group than in the infertility group ($p<0.05$). Dense-type adhesions were more common in the infertility group than in the pregnancy group ($p<0.05$).

Conclusion: According to the localization and types of adhesions, fundal and dense-type adhesions are among the features of uterine factor-related infertility. However, isthmus-located and film-type adhesions may not cause infertility.

Keywords: Intrauterine adhesion, Asherman's syndrome, infertility, hysteroscopy, pregnancy

Öz

Amaç: İntrauterin adezyon (İUA), uterin girişimlere bağlı olarak endometriyal kavitede bant şeklinde fibrotik dokuların oluşmasıdır. Çoğu adezyon, asemptomatik kalarak fertiliteyi ve gebelik koşullarını etkilemez. Ancak bazı kadınlarda infertiliteye ve olumsuz gebelik sonuçlarına neden olabilir. Bu çalışmanın amacı, hangi lokalizasyondaki ve hangi tipteki İUA'nın infertiliteye neden olabileceğini belirlemektir.

Gereç ve Yöntemler: İUA tespit edilen 76 kadına ait veriler, geriye dönük olarak tarandı. İnfertil kadınların grubuna, uterin faktöre bağlı infertilitesi olan 39 kadın dahil edildi. Gebe kadınların grubuna ise ikinci trimester ultrasonografide intaruterin adezyonları olan ve miadında sezaryen ile canlı doğum yapan 37 gebe kadın dahil edildi. Adezyonların lokalizasyonları; fundus, korpus, isthmus ve cornu olarak belirlendi. Adezyonlar tipine göre dens ve film tip olarak sınıflandırıldı.

PRECIS: To evaluate the effects of adhesion localization and types on fertility, we compared the adhesions of women who had a live birth at term with those of women who received infertility treatment.

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Bulgular: Adezyonların tipi ve lokalizasyonuna göre infertil kadınların grubu, gebe kadınların grubu ile karşılaştırıldı. İnfertil kadınların grubunda, fundusda lokalize olan adezyonlar, gebe kadınların grubuna göre daha sık izlendi. İki grup aradaki fark istatistiksel olarak anlamlıydı (p<0,05). Ancak istmus yerleşimli adezyonlar, gebe kadınların grubunda infertil kadınların grubuna kıyasla daha sık olarak gözlendi (p<0,05). Dens tip adezyonlar ise, infertilite grubundaki kadınlarda, gebe gruptaki kadınlara göre daha sık gözlendi (p<0,05).

Sonuç: Lokalizasyon ve tipine göre, fundus yerleşimli ve dens tip adezyonlar, uterin faktöre bağlı infertilitenin önemli nedenleri arasındadır. Ancak istmus yerleşimli ve film tipteki adezyonlar ise infertiliye neden olmayabilir.

Anahtar Kelimeler: İntrauterin adezyon, Asherman sendromu, infertilite, histeroskopi, gebelik

Introduction

Intrauterine adhesions (IUA) and Asherman's syndrome are terms that are often used interchangeably. IUA is identified as an increase in fibrotic tissue in the endometrial stromal and glandular alterations, which renders the functional and basal layers of the endometrium indistinguishable[1]. Risk factors include the intrauterine processes that can damage the basalis layer, such as pregnancy, intrauterine procedures, infection or inflammation, and uterine compression suture[2]. Hysteroscopy is useful for diagnosis, classification, and treatment[3-5]. Adhesions spread are not limited to specific regions in the intrauterine cavity, instead, they may be observed not only in a specific localization of the intrauterine cavity such as the isthmus or fundus but also in the entire uterine cavity[6].

Parameters such as grade, location, the severity of symptoms, appearance, prognosis, and postoperative outcome are used in the IUA classification[4,7-10]. The last classification in 2017 on IUA was developed based on the prognosis of the IUA, in collaboration with the European Society of Gynaecological Endoscopy (ESGE)[3,9,10].

IUA presents an increased rate of infertility, poor implantation, and abortion[11,12]. The IUA-related infertility frequency is reported to be 43% and is distinguished by poor sperm motility and/or absence of implantation[13]. The possible complications in pregnant women with IUA are spontaneous abortion, intrauterine growth restriction, preterm delivery, placenta accreta, presentation abnormalities, or placenta previa[14-16].

In this study, we determined the locations and type of IUA which are associated with uterine factor-related infertility.

Materials and Methods

This study was designed as a retrospective study and the patient data was retrieved from previous patient files from VM Medicalpark Kocaeli Private Hospital. The study was approved by the Kocaeli University Non-invasive Clinical Research Ethics Committee with the project number of 2017/305 and the reference number of KU GAKAEK 2017/15.7. All methods were carried out following the relevant guidelines and regulations.

Signed informed consent was obtained from all patients who were admitted to VM Medicalpark Kocaeli Hospital before the hysteroscopy and the routine ultrasonography (USG) for second-trimester abnormality screening.

The study was composed of 76 women with adhesions who met the study inclusion criteria. Thirty-nine women with a uterine factor-based infertility history for at least one year were assigned to the infertility group. However, any IUA-unrelated infertility phenotypes were excluded from the study. Thirty-seven women suspected of adhesions in the second-trimester USG and who had a live birth via cesarean section at term were assigned to the pregnancy group of the study. This pregnancy group of patients had no recurrent pregnancy loss history or any fetal abnormalities. Amniotic fluid index, cervical length (>35 mm), and placentation was within the normal range according to the USG date (placenta acreata was not observed). The suspicions of adhesion in the second trimester USG of the women in this group were confirmed intraoperatively during cesarean section. The IUA in the infertility group was diagnosed by a filling defect on hysterosalpingography and hysteroscopy. The adhesion in the pregnancy group was incidentally diagnosed during the second-trimester USG screening and confirmed intraoperatively during cesarean section for the presence of any abnormality. The IUA of the women were evaluated by a single specialist with a similar procedure, using the same hysteroscope and USG instrument. In the second trimester USG, adhesions were previously defined by the same specialist as "Sheet on string"[17] (Figure 1).

The IUA is classified into four distinct groups according to their localization isthmus, corpus, fundus, and corns. According to their structures, synechiae are classified as film or thick adhesions.

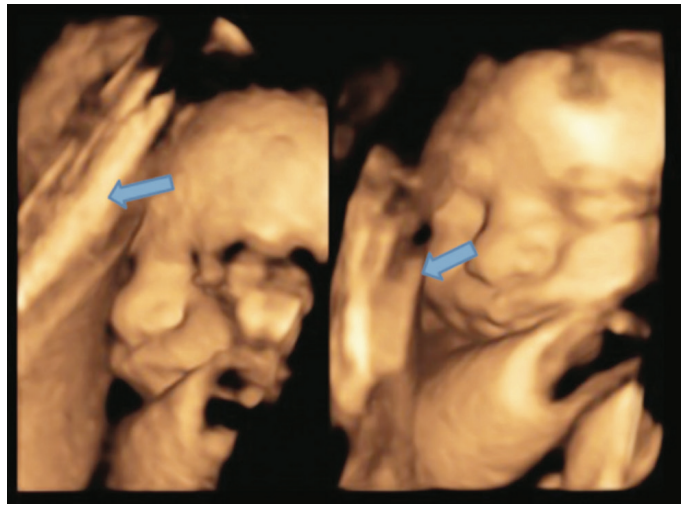

Figure 1. Intrauterine adhesion demonstrated using 4D ultrasonography; "sheet on a string" appearance of the pregnancy group[17]

Statistical Analyses

Retrospective data were statistically evaluated with the Statistical Package for the Social Sciences program (version 20). Data were collected as mean and ± standard deviation of quantitative variables. Frequency and percentage values were used to summarize the qualitative variables. The odds ratio and 95% confidence interval were calculated for each result. The group comparisons were performed using the chi-square test and Student's t-test for qualitative variables. A p-value of <0.05 was considered statistically significant.

Results

The women with IUA were divided into 2 groups according to the imaging methods of the adhesions. The diagnosis of adhesions in the infertility group was made by hysteroscopic procedure and the diagnosis of adhesions in the pregnancy group was made by the second trimester USG. For both groups of patients, we recorded age, body mass index, number of gravidities, number of parity, number of miscarriages, number of menstrual regulation, presence of prior infertility treatment, and prior cesarean section (Table 1). Statistically, a significant difference was observed among all the whole demographic data except for the body mass index and number of gravidity.

Figures 2 and 3 depict the localizations and types of adhesions in both groups. The dense-type and fundal adhesions of the infertility group and the film-type and isthmic adhesions of the pregnancy group are shown in the figures.

The results of the study are given in Table 2. The dense-type adhesions were found to be more common than the film-type adhesions in the infertility group (p=0.006). In the pregnancy group, however, the film-type adhesions were more frequent than dense-type adhesions (p=0.006). Adhesions were also evaluated in terms of their localization. Among the adhesions in the infertility group, those localized in the fundus were more common than those localized in the isthmus, corns, and corpus [n=27 (69.2%)]. Besides, the adhesions of the pregnancy group localized in the isthmus were observed more frequently than adhesions localized in the fundus, corns, and corpus [n=20 (54.1%)].

The types and localizations of the adhesions were compared among the groups. According to our findings, dense-type adhesions were observed to be more frequent in the infertility group compared with the pregnancy group (p<0.05). As the frequency of film-type adhesions was compared, film-type adhesions were observed to be more common in the pregnancy group than in the infertility group (p<0.05). As the localization

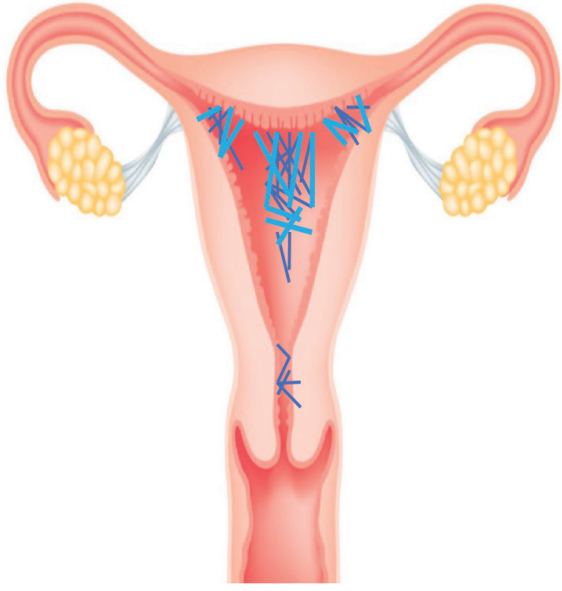

Figure 2. Intrauterine adhesions of the infertility group

Table 1. Baseline characteristics of the participants

Characteristic	Infertility group (n=39)	Pregnancy group (n=37)	p-value
Age (year) mean ± SD§	30.4±3.2	27.8±4.3	0.003
BMI§ (kg/m²) mean ± SD	26.1±4.9	25.2±5.3	0.39
Number of gravidity mean ± SD	2.79±1.1	2.89±0.7	0.66
Number of parity mean ± SD	0.33±0.57	0.70±0.46	0.003
Number of miscarriages mean ± SD	1.94±1.2	0.78±0.7	<0.001
Number of menstrual regulation mean ± SD	0.1±0.3	0.4±0.5	0.002
Prior infertility treatment	28 (71%)	5 (13%)	<0.001
Prior cesarean section	6 (15%)	21 (56%)	<0.001

SD§: Standart deviation, BMI§: Body mass index, a p-value of <0.05 was considered statistically significant

of the adhesions was evaluated, fundal localization was found to be more common in the infertility group than the pregnancy group (p=0.001); and the prevalence of isthmic and cornual localizations was higher in the pregnancy group compared to the infertility group [Table 2, (p<0.05)].

Discussion

The IUA can emerge with noticeable symptoms, asymptomatic symptoms, or mainly with the complaint of infertility/subfertility, which is the most prevalent presentation of IUA[18,19].

The hysteroscopy is the gold standard diagnostic method of IUA[3,20]. The last ESGE classification in 2015 was based on the prognosis of the IUA[9,10,21]. However, there is no study in the literature reporting the relationship between infertility and IUA localization[3,10,22,23]. Tubal and isthmus but not fundus and corpus-localized adhesions are included in calcifications[3,10].

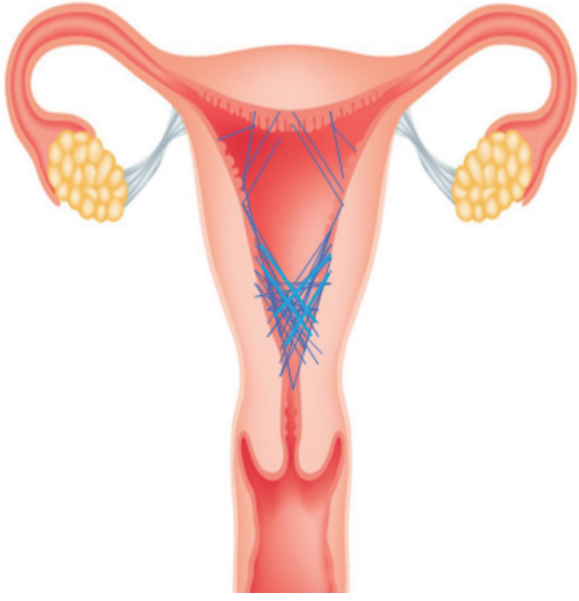

Figure 3. Intrauterine adhesions of the pregnancy group

As we have hypothesized that the type and localization of adhesions may affect uterine factor-related infertility, we retrospectively compared the results of hysteroscopy for diagnosis and treatment of women with uterine factor-related infertility and the results of the second-trimester USG screening of pregnant women with IUA for the presence of any abnormality.

In the infertility group, any IUA-unrelated infertility phenotypes were excluded from the study to evaluate the net effects of adhesions. Women with adverse pregnancy outcomes (such as fetal anomaly, placenta accreta, and preterm birth) were excluded from the study in the pregnancy group for similar reasons. The fact that hysteroscopy procedures and second-trimester USG were conducted by the same expert is one of the major features that helped standardize the outcomes of this study.

We observed that the adhesions in the fundal localization were more frequent in the infertility group than in the pregnancy group. This finding supports the idea that the fundus-localized adhesions have a negative impact on fertility. In a survey conducted by Feng et al.[14] in 1999, the authors concluded that if the overall area of the endometrium changes by the presence of increased adhesions, fetal improvement would is negatively affected due to decreased vascularization. In parallel with these findings, we found that dense-type adhesions were more common in the infertility group. According to this outcome, when the area in the endometrium influenced by adhesions increases, fetal improvement is negatively impacted due to decreased vascularization. Fundal adhesions, probably, are considered as infertility leading factor due to restraining the implantation and growth of the fetus[15]. In a previous essential study, isthmic adhesions were concluded to have roles in infertility by inhibiting sperm motility from fundus to fallopian tubes[6]. However, there is no study in the literature validating the results of this study.

In this study, the presence of amniotic fluid in the second trimester USG enabled us to easily detect adhesions like saline in

Table 2. The results of hysteroscopic procedure in the infertility group and the results of second trimester ultrasonography in the pregnancy group

	Infertility group	Pregnancy group	p-value
Type of adhesions			
Rate of film adhesions	23 (59%)	33 (89.2%)	0.006
Rate of dense adhesions	16 (41%)	4 (10.8%)	0.006
Ratios of adhesions according to their localization			
Isthmus	3 (7.7%)	20 (54.1%)	0.001
Fundus	27 (69.2%)	1 (2.7%)	0.001
Corpus	7 (17.9%)	4 (10.8%)	0.577
Cornu	2 (5.1%)	12 (32.4%)	0.002

SD: Standart deviation, a p-value of <0.05 was considered statistically significant

hysterosonography. In these women, through second-trimester USG screening, isthmic and cornual adhesions were observed more frequently than in other localizations. Considering the healthy pregnancies of these women, which resulted in term and live birth, we conclude that the isthmic and cornual adhesions may not adversely affect the formation and continuation of pregnancy. According to the IUA's most recent classifications, no studies define adhesions as film or dense.

When pregnancy coexists the presence of IUA; spontaneous abortion, intrauterine growth restriction, premature labor, placenta accreta, or placenta previa, and presentation abnormalities are to be expected[14-16].

There is not enough literature data comparing expectant management with the surgical approach, but surgery is indicated in symptomatic patients[3]. In a review of postoperatively evaluated infertile 800 patients, 60% pregnancy rate and 38.8% live birth rates were reported, whereas the live birth rate in a randomized controlled trial was 60-70%[6,13]. However, complications such as new adhesion formation due to destruction in the endometrium, uterine perforation, visceral damage if uterine perforation occurs, complications related to anesthesia, fluid overload (hyponatremia) due to distending media used, and gas embolism can also be observed after hysteroscopy[3].

Study Limitations

The limitations of the authors' study are that pregnancy outcomes after hysteroscopy were excluded from the infertility group and adhesions were not evaluated with hysteroscopy in either group. Hysteroscopy is the gold standard method for evaluating adhesions. Therefore, in future studies to determine the effects of adhesions on fertility, adhesions in randomly selected women should be evaluated by hysteroscopy and long-term fertility results should be included.

Conclusion

At this point, surgical treatment for the right indication gains importance. However, there is no data in the literature revealing the relationship between adhesion type &localization with the prognosis to determine the correct indication. This study shows that the effects of localization and types of adhesions on prognosis may be important in evaluating the current classification systems and will be important in determining the correct indications of the surgical approach. However, there is a need for more randomized controlled studies with larger numbers of patients to evaluate the effects of localization and types of adhesions on uterine factor-related infertility.

Ethics

Ethics Committee Approval: The study was approved by the Kocaeli University Non-invasive Clinical Research Ethics Committee with the project number of 2017/305 and the reference number of KU GAKAEK 2017/15.7.

Informed Consent: Signed informed consent was obtained from all patients.

Peer-review: Internally peer-reviewed.

Authorship Contributions

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

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Does hysteroscopic resection of polyps require cycle cancellation in women undergoing controlled ovarian hyperstimulation in the ICSI cycle?

ICSI döngüsünde kontrollü over hiperstimülasyonu uygulanan kadınlarda poliplerin histeroskopik rezeksiyonu döngü iptali gerektirir mi?

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Abstract

Objective: Endometrial polyps are one of the most extensive pathologies in the uterus and can be detected incidentally during assisted reproductive therapy in asymptomatic women.

Materials and Methods: In patients planned for in vitro fertilization or intracytoplasmic sperm injection (ICSI) treatment, embryo freezing, or cycle cancelation options are mandatory in many clinics when detected at the beginning of the cycle. In our study, in ICSI treatment, patients with a single endometrial polyp smaller than 1.5 cm, who underwent hysteroscopic polyp resection at the beginning of the cycle and underwent fresh embryo transfer without canceling the treatment (n=31), and patients with the same characteristics of endometrial polyp who underwent hysteroscopic polyp resection before the cycle (n=34) are compared within the pregnancy, abortion and live birth rates.

Results: As a result, no statistical difference was found between the two groups' pregnancy, abortion, and live birth rates.

Conclusion: Hysteroscopic resection of polyps during ovarian stimulation in ICSI treatment does not affect pregnancy and live birth rates and may eliminate the necessity of freezing.

Keywords: Endometrial polyp, ovarian stimulation, hysteroscopy, ICSI

Öz

Amaç: Endometriyal polipler, rahimdeki en yaygın patolojilerden biridir ve asemptomatik kadınlarda yardımcı üreme tedavisi sırasında tesadüfen saptanabilir.

Gereç ve Yöntemler: İn vitro fertilizasyon veya intrasitoplazmik sperm enjeksiyonu (ICSI) tedavisi planlanan hastalarda siklusun başlangıcında tespit edildiğinde birçok klinikte embriyo dondurma veya siklus iptali seçenekleri zorunludur. Çalışmamızda ICSI tedavisinde, siklusun başında histeroskopik polip rezeksiyonu yapılan ve tedavi iptal edilmeden taze embriyo transferi yapılan 1,5 cm'den küçük tek endometriyal polipi olan hastalar (n=31) ve aynı siklus öncesi histeroskopik polip rezeksiyonu (n=34) uygulanan endometriyal poliplerin özellikleri gebelik, abortus ve canlı doğum oranları açısından karşılaştırılmıştır.

Bulgular: Sonuç olarak iki grubun gebelik, abortus ve canlı doğum oranları arasında istatistiksel olarak fark bulunmadı.

Sonuç: ICSI tedavisinde yumurtalık uyarımı sırasında poliplerin histeroskopik rezeksiyonu, gebelik ve canlı doğum oranlarını etkilemez ve dondurma gerekliliğini ortadan kaldırabilir.

Anahtar Kelimeler: Endometriyal polip, over stimülasyonu, histeroskopi, ICSI

PRECIS: There is no significance between pregnancy, abortion, and live birth rates of patients who underwent hysteroscopic polyp resection without cycle cancelation during ovarian stimulation.

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Introduction

One of the most common structural pathologies that can cause low implantation in the uterine cavity is endometrial polyps[1]. Their number and size may differ. They can be found in the uterine cavity with or without a stalk. These lesions, usually caused by the overgrowth of endometrial glands and stroma, consist of three layers; endometrial glands, stroma, and blood vessels[2]. Polyps can be asymptomatic most of the time[3], but when they are symptomatic, they most commonly present with abnormal uterine bleeding[4], and they may cause infertility at a lesser rate[5].

Endometrial polyps are found in 5-10% of infertility and 15-50% of recurrent miscarriages[6-8]. Endometrial polyps that can be noticed in the evaluation of abnormal bleeding can only be diagnosed during infertility examination[9]. The probability of their transformation into malignancy is low[10,11].

In the hysteroscopic evaluation, endometrial polyps have been found in up to 25% of women with unexplained infertility[9]. In diagnostic hysteroscopy studies performed before in vitro fertilization (IVF) treatment, the incidence of endometrial polyps in asymptomatic women was reported to be 6-30%[12-14]. The appearance of an incidental polyp during ovarian stimulation in IVF or intracytoplasmic sperm injection (ICSI) treatment cycles can put the clinician in a difficult position. In some retrospective studies, polyps have been associated with recurrent miscarriage and infertility[6,8]. Any structural pathology in the uterine cavities, such as fibroids, polyps, intrauterine adhesions, endometritis, or the presence of reduced endometrial thickness, may lead to low pregnancy rates. It has been suggested that endometrial polyps negatively affect implantation by impairing receptivity. In a case-control study, the levels of HOXA 10 and HOXA 11 mRNA, which are markers of endometrial receptivity, were measured, and a decrease in these marker levels was shown in the presence of endometrial polyps[9].

Endometrial polyps are the most common lesions affecting the endometrial cavity[15]. Endometrial polyps have been proven to interfere with fertility with both natural pregnancy[16-18] and intrauterine insemination[19]. In the presence of endometrial polyps during IVF or ICSI: (i) the cycle can be canceled, and polypectomy can be performed; (ii) the cycle can be continued and the resulting embryos scheduled to be frozen for embryo transfer a few months later; (iii) polyp can be ignored (iv) hysteroscopic polypectomy can be performed without cycle cancelation[20,21]. The variety of these treatment options can confuse assisted reproductive clinicians who aim for better implantation and pregnancy rates.

There are a few studies investigating the effect of endometrial polyps on IVF/ICSI cycles. Isikoglu et al.[1] reported that endometrial polyps smaller than 1.5 cm discovered or before IVF/ICSI cycles do not affect implantation and pregnancy rates. Lass et al.[22] found that polyps smaller than 2 cm did not decrease pregnancy rates, but increased miscarriage rates. Therefore, they argued that freezing all embryos after oocyte retrieval followed by hysteroscopic polypectomy produced better baby-to-home rates, and they suggested the possible functional approach of "losing" a few months. Also studies performed hysteroscopic polypectomy with stimulation before oocyte retrieval without cycle cancelation[20,21]. Our study compares the pregnancy, abortion, and live birth rates of patients who underwent hysteroscopic polyp resection without cycle cancelation during ovarian stimulation with those who underwent polyp resection before stimulation. In this way, the necessity of canceling the cycle due to endometrial polyps, which can be seen frequently in women receiving ICSI treatment, will be questioned.

Materials and Methods

Ethics committee approval was obtained for this study by Haliç University Ethics Committee (2022/48). In this retrospective study, electronic data of 65 women who applied for private examination between 2017 and 2020, whose ICSI processes were performed at Haliç Hospital, and whose all follow-up, treatment, and hysteroscopic operations were performed by a single physician were included in the study. Endometrial polyps seen during controlled ovarian stimulation (COS) protocol in 31 patients were resected using cold scissors with a 2.9 mm 30 degree office hysteroscope (Storz, Germany) under general anesthesia at the latest on the 10th day of the cycle. This group is group 1.

In 34 patients, the endometrial polyp detected at the time of application for treatment was resected hysteroscopically with the same method in the cycle before the start of COS treatment. These patients were in group 2. All patients were 38 years of age or younger, ICSI treatment was applied after ovarian stimulation, and the resulting embryos were cultured until the blastocyst stage. All the patients were patients with a normal response, high response patients at risk of ovarian hyperstimulation syndrome (OHSS), patients with low ovarian reserve or low ovarian response were excluded from the study. The study excluded patients with multiple polyps or polyps larger than 1.5 cm. Additionally, those with a uterine anomaly, those with a known chronic disease, those treated for excessive male factor, spouses of azoospermic men, and patients with endometriosis were excluded from the study. The same COS protocol was applied to all patients. A single blastocyst embryo transfer was performed on the fifth day of the same cycle by providing the same luteal phase support to all of them. The pregnancy test was performed with a serum BhCG test on the 12th day after embryo transfer. Clinical pregnancy was recorded as positive with the detection of fetal cardiac activity in transvaginal ultrasonography at the 6th week. Losses up to the 20th week of pregnancy is considered an abortion. Deliveries occurring toward the 37th week of pregnancy were accepted as the term. The data of all pregnant and non-pregnant patients were recorded electronically, and the follow-up of the data continued until delivery. All patients signed the informed

consent form that they read before starting IVF treatment. Within this form, they accepted and approved the use of their medical data in scientific studies. The primary outcome of our study was to compare abortion and live birth rates between the groups. Secondary outcomes are pregnancy, abortion rates, and embryo implantation rates.

Ovarian Stimulation Protocol

After the gynecological examination and transvaginal ultrasonography performed on the second/third day of menstruation in all patients included in the study, the gonadotropin dose was determined on the basis of age, body mass index (BMI), antral follicle count, basal follicle-stimulating hormone (FSH) value, and ovarian response data obtained in previous trials. For this purpose, subcutaneous injection of recombinant FSH (Gonal F, Merck Serono, Switzerland) started with a maximum of 375 IU. The patients were monitored by transvaginal ultrasonography every 2 days after the first 4 days. When the leading follicle reached 13-14 mm, Cetrotide 0.25 (Merck Serono, Switzerland) added to the treatment as a GnRH antagonist. Hysteroscopic polyp operations of the group 1 patients, whose endometrial polyps were detected during the serial evaluations, were performed until the 10th day of the cycle at the latest. When the leading follicle reached 17-18 mm, recombinant hCG (Ovitrelle, Merck Serono, Switzerland) applied subcutaneously for final maturation, and oocyte retrieval (OPU) was performed 36 h later.

OPU and ICSI and Embryo Transfer Procedures

OPU was performed in all patients with a double-lumen 17G needle in the lithotomy position under sedative anesthesia. After denudation, ICSI was applied to the oocytes retrieved after the procedure after 2-3 hours of incubation. Fertilization was confirmed with the pronucleus control performed after 17 h, and the retrieved embryos were cultured until the 5th day. Fresh, single blastocyst transfer was performed in all patients. Anesthesia was not applied in embryo transfer procedures. All transfers were performed in the lithotomy position with the bladder full, accompanied by abdominal ultrasonography.

Hysteroscopic Polypectomy

The patients were covered with sterile drapes after cleaning the vulva and vagina in the lithotomy position under general anesthesia. The cervical os was visualized by placing the speculum. Without dilating the cervical os, the cavity was entered with a 30-degree optical hysteroscope (Storz, Germany) with a shaft thickness of 2.9 mm. Physiological saline was used for cavity expansion. The polyp in the cavity was cut from the stem using cold scissors, gently taken out of the cavity with the help of forceps and sent for pathological examination. Before the procedure was completed, the presence of other lesions or masses, the presence of septum and tubal ostia were checked to ensure a normal anatomical structure. During this process, care was taken to create the least possible contact with the endometrial tissue and not create trauma.

Luteal Phase Support

Vaginal progesterone (Crinone gel 8%, Merk Serono, Switzerland) twice a day was started in all patients for luteal phase support after the OPU procedure. On the same day, 4 mg estradiol (Estrofem TB, Novo Nordisk, Denmark) was added to the treatment. Luteal phase support was continued until the 9th week of pregnancy.

Statistical Analysis

Based on the data of Fatemi et al.'s study[13], it was decided to take 30 patients for each group with 80% power and 5% margin of error. Demographic data and frequency of clinical findings are presented together with frequency and descriptive statistics. In continuous variables, data were given as a median and interquartile range. Comparisons between groups were made with the Mann-Whitney U test. The chi-square test was used to compare categorical variables between the groups. A value of $p<0.05$ was considered statistically significant. IBM SPSS 25.0 was used for statistical analyzes.

Results

The comparison of baseline values such as age, BMI, basal FSH, and treatment indications of group 1 and group 2 is presented in Table 1. Accordingly, no statistical difference was observed between group 1 and group 2 in baseline values. Stimulation time, total FSH dose, serum estradiol, and progesterone values on hCG day and endometrial thickness value data on hCG day obtained during the COS of all patients are presented in Table 2 comparatively. In the same table, the number of oocytes retrieved in OPU, the number of metaphase 2, and the number of fertilized oocytes after ICSI are also compared. Table 2 shows that these values were not statistically different between the groups. When the clinical results were compared, a single embryo transfer was performed in all patients in both groups. There was no significant difference between the groups regarding embryo implantation rates (p=0.457). Pregnancy test positivity did not show a statistical difference in both groups (p=0.457). The clinical pregnancy rate was 58.1% in group 1 and 55.9% in group 2 (p=0.859). When the abortion rates were evaluated, there was no difference between the groups (p=0.924). While the live birth rate was 51.6% in group 1, it was 50% in group 2, and there was no statistical difference between the groups (p=0.897) (Table 3).

Discussion

Endometrial polyps are one of the most common endometrial pathologies, and they may cause interruption of treatment or change of treatment method in infertile patients treated with assisted reproductive methods. According to the Cochrane database, untreated endometrial polyps have been associated infertility and subfertility[24-26].

Table 1. Baseline values

	Group 1 (n=31)	Group 2 (n=34)	p-value
Age	30.0 (25.0-32.5)	31.0 (26.0-33.0)	0.49*
Basal FSH (IU/L)	7.5 (6.1-8.4)	7.15 (5.77-9.13)	0.98*
BMI (kg/m^2)	26.8 (22.7-28.7)	27.1 (24.0-31.03)	0.49*
Indication % (n)			
Male factor	41.9 (13)	26.5 (9)	0.27**
Tubal factor	19.4 (6)	35.2 (12)	
Unexplained infertility	38.7 (12)	38.2 (13)	

Values are median unless otherwise noted (interquartile range). FSH: Follicle-stimulating hormone, BMI: Body mass index. *Mann-Whitney U test, **Chi-square test

Table 2. COS values

	Group 1 (n=31)	Group 2 (n=34)	p-value*
Stimulation time	9 (9-11)	10 (9-11)	0.37
Total FSH dose (IU/L)	1975 (1612-3100)	2901 (1893.3-3381.3)	0.16
hCG day serum estradiol level (pg/mL)	1775 (1278-2244)	1770.5 (1508.3-2402.3)	0.39
hCG day serum progesterone level (ng/mL)	0.8 (0.6-0.9)	0.8 (0.6-0.9)	0.921
hCG day endometrial thickness (mm)	10.0 (9.4-10.7)	9.9 (9.1-11.9)	0.95
Number of oocytes retrieved	12 (8-15)	11 (8-14)	0.57
metaphase II oocyte number	9 (5-13)	7 (5-11)	0.22
Number of 2 PN fertilized oocytes	7 (4-11)	5 (4-8)	0.37

Values are median unless otherwise noted (interquartile range). COS: Controlled ovarian stimulation, FSH: Follicle-stimulating hormone, hCG: Human chorionic gonadotropin. *Mann-Whitney U test

Table 3. Clinical results

	Group 1 (n=31)	Group 2 (n=34)	p-value
Number of embryos transferred*	1 (1-1)	1 (1-1)	1**
Embryo implantation rate* % (n)	67.7 (21)	58.8 (20)	0.457***
Positive pregnancy test % (n)	67.7 (21)	58.8 (20)	0.457***
Clinical pregnancy % (n)	58.1 (18)	55.9 (19)	0.859***
Abortion %(n)	6.4 (2)	5.8 (2)	0,924***
Live birth % (n)	51.6 (16)	50 (17)	0.897***

*Values are average (interquartile range), **Mann-Whitney U test, ***Chi-square test

Polyps can be associated with infertility, blocking the treatment process and causing a negative process for the patient.

It is thought that polyps prevent implantation by narrowing the available space in the endometrial cavity or by triggering inflammatory processes, or impairing receptivity[9]. Many studies in the literature agree on removing polyps before embryo transfer. Scientific evidence shows that 63% of patients reach pregnancy after removing polyps[24-26].

Again, Cochrane data showed that when the polyps detected incidentally during IVF cycles are removed hysteroscopically after the embryos are frozen, pregnancy success is similar to that in the fresh cycle[23,25,27,28].

Few studies have attempted the removal of polyps during COS and embryo transfer in the same cycle. Among them, Batioglu's study[21] presents a single patient, while Madani et al.'s study[23] includes the analysis of 9 patients. Although the numbers in these studies are insufficient, the results show that polyp removal during COS does not affect the pregnancy rate, as in our study.

Our study shows that hysteroscopic polyp resection during COS in patients scheduled for ICSI treatment does not affect pregnancy, abortion, and live birth rates. While other studies in the literature show similarities that pregnancy rates do not change, the study by Tiras et al.[29] also shows that the optimal timing for polyp resection or before COS does not change live birth rates.

In ART cycles, the cancelation of the cycle for any reason creates emotional stress for the patient and her partner[29]. Although studies show the superiority of frozen embryo treatments, there is no clear consensus on this issue[30,31]. The cost of treatment will increase due to the total unnecessary freezing[32].

A good transvaginal ultrasonographic evaluation in a patient with an endometrial polyp may be sufficient to detect endometrial polyps without the need for an additional imaging method[33]. If the polyp is single and smaller than 1.5 cm, hysteroscopic polyp removal is be an approach that should be considered a good alternative while COS treatment continues. When the patient is informed that pregnancy and live birth rates will not be affected by this procedure, total freezing will only be an approach that should be considered in the presence of hyperstimulation (OHSS) risk and high progesterone values. In this way, the cost of treatment and the patient's emotional stress can be reduced.

Study Limitation

The limitations of the study are that it is retrospective, the number of patients is small, and there is no long follow-up period.

Conclusion

Hysteroscopic resection of polyps during ovarian stimulation in ICSI treatment does not affect pregnancy and live birth rates and may eliminate the necessity of freezing.

Ethics

Ethics Committee Approval: Ethics committee approval was obtained for this study by Haliç University Ethics Committee (2022/48).

Informed Consent: Retrospective study.

Peer-review: Externally and internally peer-reviewed.

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

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Clinical significance of serum and follicular fluid ceramide levels in women with low ovarian reserve

Serum ve foliküler sıvı seramid düzeylerinin düşük over rezervli kadınlarda klinik önemi

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Abstract

Objective: Ceramide (CER) is a bioactive component of the mitochondrial membrane. In this study, we will investigate the clinical importance of serum CER (sCER) and follicular fluid CER (ffCER) levels in the lipid synthesis pathway and their effect on poor oocyte quality and in vitro fertilization (IVF) outcome.

Materials and Methods: This cross-sectional, case-control study was conducted in the IVF unit of a maternity hospital in the capital of Turkey, Ankara. A total of 88 women undergoing their first IVF cycle were included in this study patients were divided into 2 groups according to current diagnostic criteria for their ovarian reserves. Baseline sCER levels, and ffCER concentrations retrieved on the oocyte pickup day were measured.

Results: The mean age, body mass index, and infertility duration of the patients was similar between the groups (all p>0.05). There was also no significant difference in the clinical pregnancy rates (38.6% vs. 47.7%, p=0.127). sCER (15.6±6.5 vs. 23.5±8.9) and ffCER (82.5±34.3 vs. 116.4±46.5) levels were statistically significantly lower in the low ovarian reserve (LOR) group (both p<0.001). The performed receiver operating characteristic curve analysis revealed that sCER and ffCER levels could predict both LOR and pregnancy.

Conclusion: This is the first study evaluating the sCER and ffCER levels of patients undergoing IVF treatment. CER may be used as an ovarian reserve markers and a biomarker capable of predicting IVF outcomes.

Keywords: Ceramid, controlled ovarian stimulation, in vitro fertilization, ovarian reserve marker, pregnancy

Öz

Amaç: Seramid (SER), mitokondri zarının biyoaktif bir bileşenidir. Bu çalışmada, lipid sentez yolundaki serum SER (sSER) ve foliküler sıvı SER (ffSER) düzeylerinin klinik önemi ve bunların kötü oosit kalitesi ve klasik tüp bebek (in vitro fertilization - IVF) sonuçları üzerindeki etkisi araştırılacaktır.

Gereç ve Yöntemler: Bu kesitsel olgu-kontrol çalışması Türkiye'nin başkenti Ankara'da bir kadın doğum hastanesinin tüp bebek ünitesinde yapıldı. İlk IVF döngüsüne giren toplam 88 kadın bu çalışmaya dahil hastalar over rezervlerine göre güncel tanı kriterlerine göre 2 gruba ayrıldı. Bazal sSER seviyeleri ve oosit toplama gününde alınan ffSER konsantrasyonları ölçüldü.

Bulgular: Hastaların yaş ortalaması, vücut kitle indeksi ve infertilite süreleri gruplar arasında benzerdi (tümü p>0,05). Klinik gebelik oranlarında da anlamlı bir fark yoktu (%38,6'ya karşı %47,7; p=0,127). sSER (15,6±6,5'e karşı 23,5±8,9) ve ffSER (82,5±34,3'e karşı 116,4±46,5) seviyeleri düşük yumurtalık rezervi (low ovarian reserve - LOR) grubunda istatistiksel olarak anlamlı derecede düşüktü (her ikisi de p<0,001). Gerçekleştirilen alıcı işletim karakteristiği analizi, sSER ve ffSER düzeylerinin hem LOR'yi hem de gebeliği öngörebileceğini ortaya koydu.

Sonuç: Bu, tüp bebek tedavisi gören hastaların sSER ve ffSER düzeylerini değerlendiren ilk çalışmadır. SER, yumurtalık rezerv belirteci olarak ve IVF sonuçlarını tahmin edebilen bir biyobelirteç olarak kullanılabilir.

Anahtar Kelimeler: Seramid, kontrollü ovaryan stimülasyon, tüb bebek, over rezerv markerı, gebelik

PRECIS: Reduced ceramide level is associated with low ovarian reserve and may predict pregnancy in IVF treatment.

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Introduction

Women's fertility reaches its peak in the early 30s and gradually declines and disappears at menopause due to a combination of several factors[1]. However, decreased fertility rates in aging women are mainly due to the reduced quality of aging oocytes, which indicates chromosomal, morphological, and functional abnormalities[2]. The number of applications to in vitro fertilization (IVF) clinics due to low ovarian reserve (LOR) is gradually increasing mainly because of factors such as social reasons (career planning and delaying childbirth), previous ovarian surgery, exposure to radiotherapy and chemotherapy, genetic reasons [such as *Fragile-X mental retardation-1* gene premutation and bone morphogenetic protein 15 (BMP-15) gene mutation], and smoking. Postponing childbearing reduces fecundity and increases the risk of infertility in women. Research has shown that lower than 5% of women with LOR can conceive[3]. Various adjuvant treatments are used in IVF cycles of patients with LOR. However, the place of these treatments in the perspective of evidence-based medicine is still controversial.

LOR can be described as reduced number, quality, and reproductive potential of oocytes. It is important to define LOR as part of the initial infertility assessment as women increasingly present for diagnostic infertility evaluation at a later age. Although many international guidelines suggest various definitions, there is no ideal test to evaluate ovarian reserve. Some ovarian reserve tests [such as Anti-Müllerian hormone (AMH), antral follicle count (AFC), and follicle stimulating hormone (FSH)] are used in clinical practice, but a single test that can reliably predict pregnancy potential has not yet been introduced[4]. Although much convincing evidence indicates that woman's chronological age is the most important determinant for IVF success, the relationship between the age and reproductive capacity can be quite variable[5]. Therefore, given the high cost and possible negative outcomes of IVF, investigating some parameters that can be used as predictive markers, particularly in women undergoing IVF due to LOR, is of great importance.

Ceramide (CER) is a bioactive component of the cell membrane. CER belongs to the phospholipid family and plays a key role in cell growth, differentiation, barrier function, migration, and apoptosis[6]. CER is formed because of the hydrolysis of sphingomyelin or the metabolism of more complex sphingolipids. It is also metabolized to form sphingosine and sphingosine 1 phosphate[7]. Sphingosine 1 phosphate and CER have many-opposing effects: Pro- and antiangiogenetic effects[8]. Recently, there have been increasing claims that the serum level of CER (sCER) and some phospholipids may be related to oocyte quality[9-11]. Some publications have reported a decrease in mitochondrial CER levels, especially in aging oocytes[12]. The synthesis and/or intracellular transport of CER, a bioactive lipid, becomes deregulated with aging. As a result, the level of CER in the mitochondria cannot reach the normal level, and this lipid imbalance decreases mitochondrial function and a negative effect on oocyte quality.

In our study, CER levels were measured for the first time in the serum and follicular fluid (FF) patients who underwent IVF treatment. In this study, we will investigate the clinical importance of CER levels in the lipid synthesis pathway and their effect on poor oocyte quality and IVF outcome.

Materials and Methods

This study was conducted in the IVF unit of the Etlik Zübeyde Hanım Training and Research Hospital Ethics Committee, between June 1, 2018, and December 31, 2018. Eighty-eight women were included in this study-half of the them were women with LOR, while the other half had mild-to-moderate male factor or tubal factor infertility. The hospital's local ethics council approved the study protocol (date/approval number: 30.05.2018/24), and written informed consent was taken from all patients who were included in the study. All the women were on their first IVF cycle, and fresh embryo transfer was applied, when applicable, without a prenatal genetic screening test.

LOR was diagnosed when a patient below 40 years had an abnormal ovarian reserve test, which is considered AFC <5-7 follicles, AMH <1.1 ng/mL, or a day 3 FSH level of more than 10 IU/L with a simultaneous estradiol (E_2) level >80 pg/mL. Male factor infertility was defined as the presence of ≥1 abnormalities in the spermiogram, according to WHO 2010 criteria. Tubal factor infertility was diagnosed after confirmation of bilateral tubal occlusion with hysterosalpingography and/or laparoscopy.

Women aged 23-39 years who were scheduled for infertility evaluation required for IVF (routine clinical examination, hormonal panel, and ultrasonographic evaluation), diagnosed with LOR, had mild/moderate male or tubal factor infertility, and had nonhemorrhagic FF were included in the study. Women or husbands who had endocrine [e.g., polycystic ovary syndrome (PCOS), diabetes, hypothalamic dysfunction, and thyroidal disorders], cardiovascular (hypertension and coronary artery disease), renal, hepatic, or immunologic diseases; had undergone pelvic surgery including the uterus or ovaries; had congenital or acquired uterine abnormalities (e.g., submucosal myoma, polyp, uterine septum, and intrauterine adhesion) diagnosed by hysteroscopy, or had severe male factor (azoospermia, severe oligoastenoteratospermia, etc.) were excluded from this study.

Biochemical parameters and baseline hormonal parameters were investigated after at least 8 h of fasting, and venous blood samples were taken by electrochemiluminescence immunoassay (Roche Diagnostics GmbH, Mannheim, Germany). All laboratory parameters, except for sCER and follicular fluid CER (ffCER) measurements, were studied on the day the blood sample was drawn. Basal (second or third menstrual day) venous blood samples for CER were separated by centrifugation

at 2,400 g for 10 min. FF samples were drawn on the day of oocyte pick up (OPU) from the single mature follicle. Collected FF samples were immediately centrifuged at 800x g for 10 min to separate the fluid from follicular cells. Serum and FF samples were kept at -80 ˚C until the working day of CER. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m^2).

Frozen samples were subsequently brought to room temperature to be dissolved, and CER values were measured using commercially available human ceramide ELISA kits (Eastbiopharm Co., Ltd., Hangzhou, PRC). The testing procedures were performed as per manufacturer's instructions. sCER and ffCER levels were calculated from a standard curve expressed as nanograms per milliliter. The intra- and interassay coefficients of variance were <10%, and the minimum detection rate was 1 ng/mL.

Controlled Ovarian Stimulation

The women were managed and monitored according to the unit's clinical protocols using 150-450 IU/day of recombinant FSH (Gonal F, Merck Serono) or purified human menopausal gonadotropin (HMG) (Merional, IBSA). Controlled ovarian stimulation (COS) was initiated on the second or third day of menstruation, and the gonadotropin doses were adjusted according to the patients' age, AFC, and BMI. The follicle monitoring was done by serum E_2 level and transvaginal ultrasound (TVUS) from the 5th day of COS, and every 1-3 days after that. An antagonist protocol was used to provide pituitary down-regulation with daily use of gonadotropin releasing hormone antagonist (Cetrotide, Merck Serono) that was initiated from the 5th or 6th day of COS when the leading follicle arrived at 14 mm. The doses of recombinant FSH/highly purified HMG were arranged according to the patients' ovarian response. Standard final oocyte maturation with 250 mcg of recombinant human chorionic gonadotropin (hCG) (Ovitrelle, Merck Serono) was triggered when two follicles reached above 18 mm or three ovarian follicles of greater than or equal to 17 mm were visible by TVUS. OPU was performed by transvaginal aspiration 34-36 h later under ultrasound guidance. For luteal phase support, vaginal progesterone (Crinone 8%, vaginal gel, Merck Serono) was applied twice daily starting from the day of OPU until pregnancy testing. Progesterone supplementation in all transfer cycles was continued until the 12th week of pregnancy for patients who conceived.

Semen Collection, Oocyte Retrieval, and Embryo Transfer

Semen samples were obtained from masturbation after a period of 2-5 days sexual abstinence. The collected semen specimens were processed using the standard swim-up technique preparation media (FertiCult™ Flushing medium, FertiPro NV) after liquefaction for 30 min at room temperature. Highly active motile sperm in the medium was carefully removed and used for the intracytoplasmic sperm injection (ICSI) procedure. The TVUS-guided oocyte retrieval procedure was executed 34-36 h after hCG triggering. Sonographic examinations and OPU procedures were carried out on all the women by the same senior clinician with has significant expertise in reproductive endocrinology. Oocyte retrieval, oocyte denudation and conventional ICSI procedures were performed in all women to rule out fertility problems. Oocytes were cultured separately in a special preequilibrated culture dish after the ICSI procedure. Throughout the culture period, a single-step medium enriched with human serum albumin (Continuous Single Culture™, Irvine Scientific, CA, USA), was used in the study. Embryo culture was performed until the 5th or 6th day at 37 °C in an air of 5% O_2, 5% CO_2, and 90% N_2, in benchtop incubators (MIRI, ESCO Medical, Singapore). Blastocysts were scored and morphologically evaluated as previously described[13]. Embryos with the best quality were chosen for transfer. A maximum of 2 embryos were transferred, and the rest were cryopreserved for future use, as there were enough good quality embryos.

Statistical Analyses

The normality distribution of the continuous variables and were tested by Kolmogorov-Smirnov test. Differences between categorical data were evaluated using the chi-square test. Student's t-test or Mann-Whitney U test was performed to compare the two independent groups. Data are shown as mean ± standard deviation, number (percentage), and median (minimum-maximum) where appropriate. Receiver operating characteristic (ROC) analysis of the area under the curve was used to determine the predictive values of CER. Spearman's correlation analysis was used to measure the strength and direction of associations between body fluids CER levels and other variables. The data were analyzed with SPSS 21.0 software (IBM Corporation, Armonk, NY, USA). A p-value <0.05 was considered as statistically significant.

Results

A total of 88 patients participated in this cross-sectional study-44 patients each in the LOR and the control groups. In the control group, 32 patients had male factor-induced infertility, whereas 12 patients had a tubal factor. All the patients included in the study underwent the IVF cycle for the first time. The mean age, BMI, and infertility duration of the patients were similar (all p>0.05). The basal FSH level was statistically significantly higher in the LOR group than in controls (9.4±1.8 vs. 6.5±1.0, p<0.001). Other baseline hormone levels, including estradiol, progesterone, luteinizing hormone, thyroid-stimulating hormone and prolactin, were similar among the groups. AFC (6.2±2.4 vs. 13.7±4.8) and serum AMH (0.7±0.4 vs. 3.0±1.2) levels, which are ovarian reserve markers, were low in the LOR group (both p<0.001). Considering the cycle characteristics of the patients, the gonadotropin dose used (2843.6±760.9 vs. 1979.1±691.2, p<0.001) was higher in the LOR group, while the peak estrogen level (1263.3±6373.8 vs. 1776.0±859.0, p<0.001) was lower, as expected. However, no statistically significant

difference was observed in endometrial thickness (9.5±3.0 vs. 10.7±3.6, p=0.426) and stimulation length (11.3±1.8 vs. 11.1±1.8, p=0.517). The number of oocytes collected and embryos obtained were higher in the control group. While fertilization rates and the number of transferred embryos were similar between the two groups, embryo quality was worse in the LOR group, and the embryos transferred on the third day were more common. A comparison of demographics, baseline hormone levels, and cycle characteristics between the LOR and control groups are provided in Table 1. Markers of lipid (low density lipoprotein cholesterol, high density lipoprotein cholesterol, very low-density lipoprotein, triglyceride, and total cholesterol) and glucose metabolism (glucose, insulin, and homeostatic model assessment insulin resistance) were similar between the two groups (Table 2). There was no significant difference in the clinical pregnancy rates (38.6% vs. 47.7%, p=0.127). sCER (15.6±6.5 vs. 23.5±8.9) and ffCER (82.5±34.3 vs. 116.4±46.5) levels were statistically significantly lower in the LOR group (both p<0.001). When the patients were categorized according to their pregnancy status, both serum and FF CER levels were found to be statistically significantly higher in the pregnant group (p<0.001, p=0.036, respectively) (Table 3). A statistically significant positive correlation was observed between basal sCER and ffCER levels both between the groups and in the whole cohort (r=0.056, p<0.001). The performed ROC curve analysis revealed that sCER and ffCER levels could predict LOR and pregnancy. A sCER level lower than 16.5 ng/mL may predict women with LOR with sensitivity of 40.9% and specificity of 70.5%, whereas ffCER level lower than 98.5% may predict the same patients with a sensitivity of 76.5% and specificity of 45.5% (Figure 1). In contrast, a sCER level higher than 18.5 ng/mL may predict pregnancy in women undergoing IVF treatment with a sensitivity of 71.1% and specificity of 74%, whereas an ffCER level higher than 121

Table 1. Comparison of demographics, baseline hormone levels, and cycle characteristics between the LOR and control groups

Variables	LOR (n=44)	Control (n=44)	p
Age (years)	30.2±4.4	30.1±5.4	0.148
BMI (kg/m²)	24.5±4.6	25.0±4.2	0.834
Infertility duration (years)	4.5±2.6	4.3±2.0	0.218
FSH (mIU/mL)	9.4±1.8	6.5±1.0	0.000
TPMSC (mil)	28.8±25.7	13.6±15.8	0.000
AFC	6.2±2.4	13.7±4.8	0.000
AMH (ng/mL)	0.7±0.4	3.0±1.2	0.000
sCER (ng/mL)	15.6±6.5	23.5±8.9	0.000
ffCER (ng/mL)	82.5±34.3	116.4±46.5	0.000
Number of oocytes retrieved	3.6±1.3	13.3±5.2	0.000
Number of M2 oocytes	3.2±1.3	10.2±4.1	0.000
Fertilization rate (years)	69.3±25.5	79.0±20.1	0.743
Number of 2PN embryos	1.9±1.0	7.8±2.8	0.000
Number of embryos	1.7±1.0	7.5±2.7	0.000
ET	1 (0-2)	1 (1-2)	0.564
Embryo quality			
FF	3 (6.8)	1 (2.3)	0.001
Grade 1	15 (34.1)	24 (54.5)	
Grade 2-3	26 (59.1)	19 (43.2)	
Transfer day			
Day 3	37 (84.1)	29 (65.9)	0.002
Day 5	7 (15.9)	15 (34.1)	
Pregnancy	17 (38.6)	21 (47.7)	0.127

LOR: Low ovarian reserve, BMI: Body mass index, FSH: Follicle stimulating hormone, TPMSC: Total progressively motile sperm count, AFC: Antral follicle count, AMH: Anti-Müllerian hormone, sCER: Serum ceramide, ffCER: Follicular fluid ceramide, ET: Embryo transfer, FF: Fertilization failure. Data were shown as mean ± standard deviation, number (percentage), and median (minimum-maximum)

may predict pregnancy with sensitivity of 50% and specificity of 80% (Figure 2).

Discussion

In this study, we assessed the baseline sCER and ffCER levels in infertile women undergoing IVF cycles due to LOR for the first time and compared them with women with normal ovarian reserve markers. CER is an important bioactive molecule located in the cytoplasm and mitochondria with different functions. We found that sCER and ffCER levels are lower in patients with LOR, and their serum and FF levels may predict IVF outcomes. Embryo quality, which is primarily determined by oocyte quality, is the most important determinant of IVF outcomes[14]. We know that AMH is closely associated with the existing

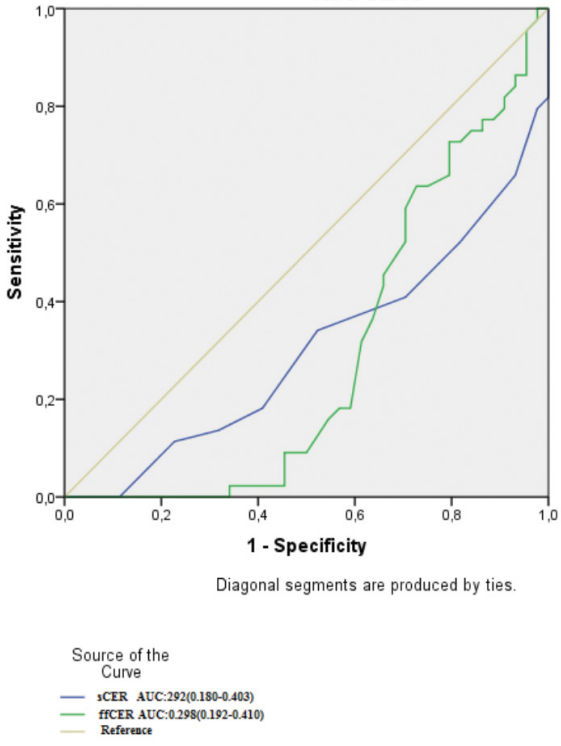

Figure 1. ROC curve analysis of sCER and ffCER level in predicting pregnancy in women undergoing IVF treatment

ROC: Receiver operating characteristic, sCER: Serum CER, ffCER: Follicular fluid CER, IVF: In vitro fertilization

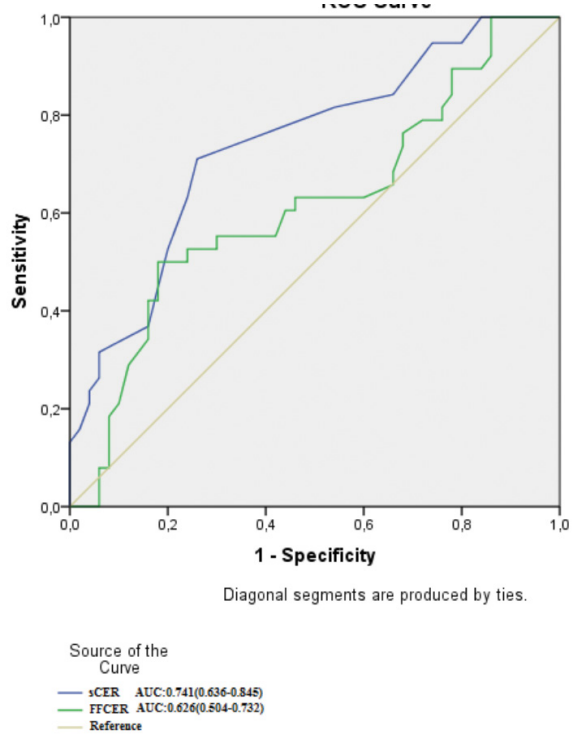

Figure 2. ROC curve analysis of sCER and ffCER level in predicting women with LOR

ROC: Receiver operating characteristic, sCER: Serum CER, ffCER: Follicular fluid CER, LOR: Low ovarian reserve

Table 2. Comparison of two groups for markers of lipid and glucose metabolism

Variables	LOR (n=44)	Control (n=44)	p
Glucose (mg/dL)	90.9±8.7	89.6±9.5	0.231
Insulin (mIU/L)	15.7±9.5	11.5±5.9	0.096
HOMA-IR	2.6±1.8	2.6±1.4	0.455
T. cholesterol (mg/dL)	171.8±30.6	169.8±29.4	0.745
LDL-C (mg/dL)	97.0±26.8	95.3±31.6	0.532
HDL-C (mg/dL)	51.3±14.9	54.5±13.2	0.367
VLDL (mg/dL)	20.2±8.6	20.0±9.1	0.889
TG (mg/dL)	99.3±46.2	101.2±42.8	0.712
T. chol/HDL	3.3±1.0	3.3±1.0	0.871

LOR: Low ovarian reserve, HOMA-IR: Homeostatic Model Assessment Insulin Resistance, LDL-C: Low density lipoprotein cholesterol, HDL-C: High density lipoprotein cholesterol, VLDL: Very low density lipoprotein, TG: Triglyceride, T. chol: Total cholesterol. Data were expressed as mean ± standard deviation

Table 3. sCER, ffCER and other ovarian reserve parameters in pregnant and non-pregnant cases

Variables	Pregnant (n=38)	Non-pregnant (n=50)	p
AMH (ng/mL)	2.0±0.9	1.8±0.7	0.223
Estradiol (pg/mL)	38.5±16.5	39.0±12.8	0.523
FSH (mIU/mL)	7.3±1.8	7.7±2	0.294
AFC	10.6±5.6	11.8±6.7	0.429
sCER (ng/mL)	21.5±5.8	16.3±6.9	0.001
ffCER	108.5±41.2	92.1±39.2	0.036

AMH: Anti-Müllerian hormone, AFC: Antral follicle count, FSH: Follicle stimulating hormone, sCER: Serum ceramide, ffCER: Follicular fluid ceramide. Data were expressed as mean ± standard deviation

ovarian reserve. The decrease in serum AMH levels due to aging is accompanied by a decline in the number of the primordial follicles, as well as increased apoptosis in the granulosa cell which indicates diminished oocyte quality. Although low-pre-treatment AMH levels in women undergoing IVF cycles indicate that the number of oocytes retrieved and oocyte quality is low, it cannot predict pregnancy. Even poor-quality embryos derived from poor-quality oocytes may result in a live birth[15].

Mitochondria are the most important energy-producing organelles of the cell and are separated from the cytoplasm by a double-layered membrane. It produces ATP, which is vital for several cellular activities. Apart from energy production, it also plays a role in the oxidation of fatty acids, calcium homeostasis, and apoptosis[16]. Poor oocyte quality and associated embryo quality may be associated with impaired energy production in the oocyte cytoplasm, although it results in live birth[17]. This energy production impairment may have different and wide range of effects from implantation to after birth.

From an evolutionary perspective, mitochondria are thought to be the remnants of bacteria that have invaded eukaryotic cells. Although many proteins necessary for its function are encoded by the nucleolus genome, mitochondria are the only animal organelles that include DNA outside the nucleus[18]. Human mitochondrial DNA (mtDNA) is a circular structure and contains 37 genes. Since it does not contain protective proteins such as histones, mtDNA is susceptible to mutations. Mitochondria play an important role in human reproduction[19]. Fertility has been shown to be severely reduced in transgenic mice with induced mtDNA mutations[20]. MtDNA is maternally inherited because sperm mitochondria are degraded after they enter the oocyte. Although the exact reason for this is unknown, it may be to protect the embryo from dangerous mutations that may occur in the mtDNA of the sperm exposed to high oxygen radicals during spermatogenesis. A fully grown oocyte has approximately 100,000 mitochondria. Since the need for ATP is also low in immature oocytes, the mitochondria that are waiting silently replicate in the late folliculogenesis stage and after fertilization. The oocytes in the mitochondria are vital for early embryonic development[21,22].

Mitochondria are essential organelles in sphingolipid metabolism, and many sphingolipid metabolizing enzymes are located in the mitochondria[12]. The presence of these pathways is an indirect indicator that lipid products also have specific functions. CER signaling, which is one of these functions, involves a complex molecular and subcellular network, all implicated in various cellular processes such as proliferation, differentiation, survival, necrosis and aging[23-26]. An experimental study showed that after the addition of a CER metabolizing enzyme, called acid ceraminidase, which is expressed in human cumulus cells and FF, to the culture medium, embryo morphology significantly improved and healthy births were achieved five-fold higher[9]. Histologically and hormonally, an experimental study showed that local ovarian CER 1 phosphate injections reduced cyclophosphamide-induced ovarian damage by protecting the ovarian reserve, restoring hormonal secretions, inhibiting apoptosis, and improving stromal vascularity. Thus, fertility, oocyte quality, and uterine morphology are protected by CER 1 phosphate[10].

Available data suggest that plasma lipoproteins, particularly high-density lipoprotein cholesterol, contain notable sfingolipids such as CER and sphingosine-1-phosphate, and they can mediate cardiovascular protection in healthy pregnancy[27]. However, we could not find any significant differences in lipid profiles of the groups. Recently, different subclasses of CER have been suggested as novel lipidomic markers for diagnosing PCOS. Similarly, some FF metabolomics, including fatty acid, di/triacylglycerol, CER, CER-phosphate, phosphatidylcholine, and sphingomyelin, have been shown to be elevated in hyper-responder women with or without PCOS undergoing IVF treatment[11]. Additionally, various growth factors in the FF have been reported to be altered according to the different ovarian responses. Vascular endothelial growth factor (VEGF) has increased in the FF of women with poor response[28]. It has also been reported that serum VEGF levels did not differ in poor responders compared to normo-responders and did not foresee ovarian response[29]. In another study, serum insulin-like growth factor-1 levels also did not differ between the poor and normo-responders[30], but a minor polymorphism

in BMP-15 that also has growth factor properties, has been shown to be related to the high response to COS[31]. We did not classify study groups based on ovarian response, but as expected, women diagnosed with LOR poorly responded to COS and had lower sCER and ffCER levels. Therefore, we may speculate that as a growth factor, CER is associated with a poor ovarian response to COS.

We showed that CER is markedly lower in patients with LOR but significantly higher in women who could conceive, unlike other ovarian reserve markers. CER also was not correlated with the other ovarian reserve markers. However, sCER and ffCER levels were well correlated. CER may play a crucial role in both the physiological and pathological processes of the ovarian folliculogenesis and may be used independently to predict pregnancy in women undergoing IVF treatment.

Study Limitations

The main drawbacks of this work are the limited sample size, the partially heterogeneous control group, the analysis of FF from only one follicle, the analysis being based on only one cycle, and the lack of cumulative pregnancy rates. Additionally, increased CER levels may be due to secretions from the liver. The endothelium may be another source of serum CER. It should be considered that oxidative stress and proinflammatory cytokines may increase endothelial CER production by activating sphingomyelinase.

Conclusion

In conclusion, this is the first study evaluating the sCER and ffCER levels of patients undergoing IVF treatment. CER may be used as an ovarian reserve marker and a biomarker capable of predicting IVF outcomes. It may also be used as a therapeutic agent in patients with LOR or poor quality of oocyte. There is a need for further investigations to reveal the involvement of CER and other sphingolipids with female reproductive functions.

Ethics

Ethics Committee Approval: The hospital's local ethics council approved the study protocol (date/approval number: 30.05.2018/24).

Informed Consent: Written informed consent was taken from all patients who were included in the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: B.T., Design: B.T., Data Collection or Processing: B.T., N.İ., İ.K., Analysis or Interpretation: O.A., Literature Search: N.İ., İ.K., S.D., Writing: B.T.

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Relationship between the follicular distribution pattern of polycystic ovaries and the degree of menstrual disturbance and serum sex steroid levels

Polikistik overlerin foliküler dağılım paterni ile menstrüel bozukluk derecesi ve serum seks steroid düzeyleri arasındaki ilişki

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Abstract

Objective: This study aimed to examine the associations between follicular distribution pattern (FDP) in polycystic ovaries and menstrual disturbances in women with infertility.

Materials and Methods: A retrospective review of patients was performed (n=73). Ultrasound images from cycle day 2-5 of a spontaneous or progestin induced menstrual cycle were reviewed. Ovaries were classified as polycystic ovarian morphology (PCOM) if they contained ≥12-follicles measuring 2-9 mm in diameter. Images of PCOM ovaries were classified as having a peripheral cystic pattern (PCP) with follicles arranged at the periphery of the ovary, or general cystic pattern (GCP) if follicles were dispersed heterogeneously throughout the ovarian stroma. Menstrual disturbance was assessed by questionnaire, and oligomenorrhea was defined as cycles >35 days in length.

Results: PCP was more strongly associated with menstrual irregularity that GCP. 94% of subjects with bilateral PCP-experienced oligomenorrhea compared with 65% of women with a unilateral PCP ovary [odds ratio (OR) 9; p<0.05]. 29% of women with bilateral GCP ovaries experienced menstrual disturbances, less than bilateral PCP (OR 36; p=0.002), but similar to unilateral PCP (OR 3; p=0.07). Serum testosterone and luteinizing hormone (LH) levels were significantly correlated with the ovarian FDP.

Conclusion: There is a relationship between menstrual irregularity or certain types of serum steroids and ovarian morphology. It remains unknown if morphology, testosterone or LH causes the menstrual disturbance or if they are co-initiated by an intervening factor.

Keywords: Polycystic ovary syndrome, oligomenorrhea, ovarian follicle

Öz

Amaç: Bu çalışmanın amacı, infertiliteli kadınlarda polikistik overlerde foliküler dağılım paterni (FDP) ile menstrüel bozukluklar arasındaki ilişkileri incelemektir.

Gereç ve Yöntemler: Hastaların geriye dönük incelemesi yapıldı (n=73). Spontan veya progestin ile indüklenen adet döngüsünün 2-5 günlerindeki ultrason görüntüleri incelendi. Yumurtalıklar 2-9 mm çapında ≥12 folikül içeriyorsa polikistik over morfolojisi (PCOM) olarak sınıflandırıldı. PCOM yumurtalıklarının görüntüleri, foliküller yumurtalığın çevresinde düzenlenmiş ise periferik kistik patern (PCP) veya foliküller yumurtalık stroması boyunca heterojen olarak dağılmışsa genel kistik patern (GCP) olarak sınıflandırıldı. Menstrüel bozukluk anket ile değerlendirildi ve oligomenore >35 gün uzunluğundaki sikluslar olarak tanımlandı.

PRECIS: In women with PCOS, there is a relationship between testosterone levels or menstrual irregularity and follicular distribution pattern, particularly when comparing the string of pearls pattern with a multicyclic distribution of follicles, spaced throughout the stroma.

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Bulgular: PCP adet düzensizliği ile GCP'den daha güçlü bir şekilde ilişkiliydi. Tek taraflı PCP yumurtalığı olan kadınların %65'ine kıyasla iki taraflı PCP'li deneklerin %94'ü oligomenore yaşamaktaydı [risk oranı (RO) 9; p<0,05]. İki taraflı GCP yumurtalıkları olan kadınların %29'u menstrüel bozukluklar yaşamaktaydı. Bu oran tek taraflı PCP yumurtalıkları olan kadınlardaki orana benzerken (RO 3; p=0,07), iki taraflı PCP yumurtalıkları olan kadınlardaki orandan düşüktü (RO 36; p=0,002). Serum testosteron ve luteinleştirici hormon (LH) seviyeleri, yumurtalık FDP ile önemli ölçüde ilişkiliydi.

Sonuç: Menstrüel düzensizlik ile belirli serum steroidleri ve over morfolojisi arasında bir ilişki vardır. Morfoloji, testosteron veya LH'nin adet düzensizliğine neden olup olmadığı veya araya giren bir faktör tarafından sürecin başlatılıp başlatılmadığı bilinmemektedir.

Anahtar Kelimeler: Polikistik over sendromu, oligomenore, yumurtalık folikülü

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disorder of unknown, multiple etiologies with a clinical syndrome characterized by hyperandrogenism, oligoamenorrhea, and infertility[1]. The clinical findings of PCOS are highly variable, making the diagnostic criteria of the condition controversial[2-4]. In 1990, the National Institutes of Health (NIH) consensus statement on diagnostic criteria for PCOS excluded ovarian morphology[5]. However, the 2003 international consensus on PCOS diagnosis held in Rotterdam proposed the inclusion of ovarian morphology into the diagnostic criteria of PCOS[6]. Although the Rotterdam criteria for polycystic ovarian morphology (PCOM) include the presence of ≥12 follicles measuring 2-9 mm in diameter and/or increased ovarian volume (>10 cm^3) in a single or both ovaries[2], there remains considerable debate over how to best define the ovarian appearance in PCOS[1]. Lujan et al.[7] reported that the follicle number per ovary (FNPO) threshold of 26 follicles resulted in the best sensitivity and specificity to distinguish women with PCOS from healthy controls. Therefore, in 2014, the Androgen Excess Society and Polycystic Ovary Syndrome Society guidelines recommended using FNPO of ≥25 for the definition of PCOM when using newer ultrasound technology with maximal ovarian follicle resolution[8]. Most recently, the 2018 international evidence-based guideline for the assessment and management of PCOS recommended using a FNPO of at least 20 follicles[9].

While the number of follicles required for the definition of PCOM has been debated and updated, there has been less discussion about the patterns of follicle distribution (FDP) within a PCOM ovary. The Adam's criteria of PCOM on ultrasound initially described 10 or more follicles arranged in a peripheral pattern around a dense core of stroma, called the "string of pearls" pattern[10]. This pattern became known as one of two classes of PCOM ovaries based on the distribution of follicles in the ovary: A peripheral cystic pattern (PCP). The second class, called a general cystic pattern (GCP), describes ovaries with multiple small follicles occupying the entire parenchyma of the ovary[11]. Takahashi et al.[12] examined differences between women with PCP and GCP ovaries and reported that serum androstenedione and the luteinizing hormone (LH)/Follicle stimulating hormone (FSH) ratio was significantly higher in women with PCP rather than GCP ovaries. This finding suggests an endocrinological difference between PCP and GCP ovaries. Furthermore, different underlying pathophysiological processes of disturbed folliculogenesis may result in different patterns of FDP in PCOM ovaries[13,14]. Earlier studies investigating the ultrasound characteristics of PCOM revealed that a peripheral distribution of ≥10 follicles around the midpoint of the ovary was a highly sensitive criterion for the diagnosis of PCOS[15].

Therefore, this study aimed to evaluate whether, in women with infertility and PCOM, the PCP FDP when measured at the ovarian midpoint is more strongly associated with menstrual irregularity compared with women GCP FDP and the relationship between FDP and serum hormone levels.

Materials and Methods

A retrospective chart review was conducted of 123 cycles of in vitro maturation (IVM) during a two-year period at the McGill University Reproductive Centre. After excluding additional cycles in patients with multiple cycles and incomplete records, 73 subjects remained for analysis. Subjects were required to have ≥12 follicles measuring 2-9 mm in diameter in at least one ovary (per the Rotterdam Criteria)[6]. Subjects with a dominant follicle ≥10 mm or an ovarian cyst on either ovary were excluded from the study. Furthermore, subjects were required to have no: Clinical or biochemical evidence of thyroid abnormalities (0.39< serum thyroid-stimulating hormone <3.0 µIU/mL); hyperprolactinemia (am fasting serum prolactin <26 ng/mL); hypothalamic pituitary dysfunction or ovarian failure (1.4< FSH >20 IU/L and estradiol >20 pg/mL); ovarian and adrenal androgen-secreting tumors (total testosterone <200 ng/mL and DHEAS <800 µg/dL); and non-classical congenital adrenal hyperplasia (am fasting 17-hydroxy-progesterone <2 ng/mL). Finally, subjects were excluded from analysis if they used hormones, clomiphene citrate, aromatase inhibitors, or other medications (including insulin sensitizing medications), which could have affected the follicle count or distribution in the previous 90 days.

All patients who underwent IVM had a baseline pelvic ultrasound by a certified technician (Quebec diplomat in Radiology techniques), and serum blood tests (total testosterone, free testosterone, LH, and FSH) on day 2-5 of a natural or progesterone provoked cycle (medroxyprogesterone, 10 mg daily taken orally for 5-14 days). The number of follicles was documented and a copy of the ultrasound images was included in the chart. Follicle count and distribution was assessed by subjective evaluation of ultrasound images by two physician investigators and images were categorized into three groups based on ovarian morphology:

1) Normal morphology: (<12 follicles) without a predominantly peripheral distribution,
2) PCP: ≥12 follicles peripherally distributed around a dense stromal core for at least 50% of the ovarian diameter.
3) GCP: ≥12 follicles located throughout the ovary and not more than 49% in a peripheral distribution.
Each physician was blinded to the other's diagnoses. If the diagnoses of the two physicians differed, a third physician was consulted and then agreement of the diagnosis of two of three physicians was then accepted. All physicians were gynecologists with extensive experience in trans-vaginal ultrasonography. The third physician was consulted only for two cases.
Menstrual regularity was determined by questioning the patient on the duration of most menstrual cycles at the time of initial presentation to the fertility clinic. Subjects with cycles less frequent than 35 days, for 75% of cycles, were considered oligomenorrheic.

Statistical Analysis

Statistical analysis was performed using Stats Direct. Chi-square tests with Yates correction and odds ratios with Fisher's exact tests were also used. ANOVA was used to compare group continuous data, while chi-squared tests were used to compare the categorical data. Tukey's Post-hoc testing was used for post ANOVA comparisons. For the case of the chi-square test, if a number was zero in one category a one was substituted. Spearman's correlation coefficient was used to compare relationships in different groupings with the continuous demographic data. Data were compared with odds ratios and confidence intervals. Data are presented as N and percentage or mean ± standard deviation.

Committee for the Protection of Human Subjects approval of the study was obtained. None of the authors have any conflicts of interest.

Results

The demographic, data, rates of menstrual disturbance and serum steroid levels, stratified for follicular distribution pattern are reported in Table 1. When considering the demographics, the groups were similar for age and BMI. The oldest woman in this study was 36 years of age. However, the group with one GCP was more likely to have conceived previously, suggesting a relationship between follicular distribution pattern and fertility potential. All subjects had follicle counts of at least twelve in one ovary.
When considering serum hormone levels (Table 1) patterns were discernable based on the follicular distribution pattern and whether that distribution pattern occurred in one or both ovaries. The serum total and free testosterone levels decreased in a linear fashion from women with two PCP, to one PCP ovary, to two GCP ovaries, then to one GCP ovaries. A similar decrease in serum LH and the LH to FSH ratio was noted in the relationship with follicular distribution patterns. These findings demonstrated statistically significant correlations between ovarian morphology and serum total testosterone ($r=-0.63$, $p<0.01$), serum free testosterone ($r=-0.58$, $p=0.01$), serum LH levels ($r=-0.66$, $p<0.01$), and the LH/FSH ratio ($r=-0.45$, $p<0.05$). (For this analysis groupings were performed in the following order two PCP, one PCP, two GCP, one GCP).
Fifty-three percent of women were oligomenorrheic (39/73), defined as having menstrual cycles longer than thirty-five days, at least 75% of the time. Sixteen subjects had PCP morphology

Table 1. Demographics, menstrual data and serum steroid levels of the patients stratified by the ovarian morphology

Ovarian morphology	2 PCP ovaries n=16 (Group 1)	1 PCP ovary n=21 (Group 2)	2 GCP ovaries n=17 (Group 3)	1 GCP ovary n=36 (Group 4)	p-value
Age (Years)	30±1.8	30±1.7	30±1.9	31±1.8	0.09
BMI (kg/m^2)	23.9±3.2	23.2±2.9	22.8±2.7	23.5±3.1	0.74
Nulliparous	16 (100%)	20 (95%)	17 (100%)	13 (36%)	0.0001
Total serum testosterone (nmol/L)	2.2±0.3	1.9±0.3	1.6±0.2	1.4±0.3	0.0001*
Serum free testosterone (nmol/L)	1.84±0.5	1.6±0.6	1.1±0.5	0.9±0.3	0.0001**
Serum LH (IU/L)*	7.9±0.9	6.7±1.0	5.7±1.3	5.5±1.2	0.0001*
Serum FSH (IU/L)*	6.5±1.2	6.5±0.9	6.7±1.4	7.2±1.4	0.13
LH/FSH ratio	1.2±0.2	1.0±0.3	0.85±0.3	0.76±0.2	0.0001***
#with Oligomenorrhea	15 (94%)	13 (62%)	5 (29%)	6 (17%)	0.0001

Note: serum bloods tests were performed on cycle day 2 to 5 of a spontaneous or Medroxyprogesterone acetate induced menstrual cycle and on the same day as the ultrasounds. PCP: Peripheral cystic pattern, GCP: General cystic pattern, BMI: Body mass index, LH: Luteinizing hormone, FSH: Follicle stimulating hormone, Data were compared using ANOVA. $p<0.05$ statistically significant.
Post-hoc testing (only statistically significant differences are indicated, $p<0.05$):
*Group 1> Group 2, Group 1> Group 3, Group 1> Group 4, Group 2>Group 3, Group 2>Group 4
**Group1>Group3, Group1>Group4, Group2>Group3, Group2>Group4
***Group1>Group3, Group1>Group4, Group2>Group4

in both ovaries, 94% (15/16) of which were oligomenorrheic. Twenty-one subjects had one ovary with PCP morphology with the second ovary having <12 follicles and 62% (13/21) of these patients were oligomenorrheic. Seventeen subjects had GCP morphology in both ovaries, 29% (5/17) of which were oligomenorrheic. Thirty-six subjects had one ovary with GCP morphology with the second ovary having <12 follicles and 17% (6/36) of them were oligomenorheic. Interestingly, none of the subjects had a PCP on one ovary and a GCP on the other ovary.

The odds ratio of menstrual disturbance comparing either of the FDPs to the other three types is presented in Table 2. PCP ovaries were more strongly associated with menstrual disturbances than were GCP ovaries. Compared to having bilateral GCP ovaries (29% oligomenorrheic), having bilateral PCP ovaries conferred 36 times increased odds of experiencing menstrual disturbance compared to women with a unilateral PCP ovary (62% oligomenorrheic), women with bilateral PCP ovaries (94% oligomenorrheic) were 9 times more likely to experience menstrual irregularities. Women with a unilateral PCP were more likely to experience menstrual irregularity (62%) than women with bilateral GCP (29%), although this was not statistically significant (p=0.07). There was no statistical difference in the rates of menstrual disturbances in women with bilateral GCP ovaries (29% oligomenorrheic) compared to women with a unilateral GCP ovary (17% oligomenorrheic). Women with a unilateral PCP ovary (62% oligomenorrheic) were 8 times more likely to experience menstrual disturbances than women with a unilateral GCP ovary (17% oligomenorrheic).

Discussion

The main objective of the current study was to evaluate whether the FDP in PCOM ovaries of women with infertility can be useful in predicting the severity of menstrual irregularities, specifically oligo and anovulation. From a clinical standpoint, it is important to understand how different PCOM morphologies are related to the severity of the disease itself. Using a subset of women undergoing IVM at the McGill University Reproductive Centre who were known to have PCOM and infertility, we evaluated whether the FDP of their ovaries correlated with the degree of menstrual irregularity experienced by the patient. We noted a significantly higher correlation between PCP ovaries and oligomenorrhea than with GCP ovaries and oligomenorrhea. Furthermore, none of the subjects in the study were found to have one ovary with each type of distribution pattern; they either had only PCP or GCP ovaries, not both.

Women with a unilateral PCOM ovary showing a PCP FDP were more likely to experience menstrual irregularity than women with bilateral GCP FDP. This finding suggests that the FDP seen in the ovary is a more significant prognostic factor for the severity of clinical presentation than is the bilaterality of PCOM ovaries. Furthermore, PCP FDP was more likely to be associated with increased total and free testosterone levels, as well as increased LH/FSH ratio. These findings are in agreement with previous studies illustrating a relationship between FDP and hyperandrogenism, supporting the assertion that PCP and GCP ovarian morphologies may differ in their endocrine and pathophysiological processes[12-14].

Christ et al.[16] previously studied the FDP and compared it with reproductive and metabolic features of PCOS to assess the use of sonographic features to predict the severity of PCOS. In contrast to the results presented in this study, Christ et al.[16] concluded that FDP was not associated with any reproductive marker or metabolic parameter associated with PCOS. A potential explanation for this discrepancy may be due to the cohort of subjects used for each study. Our study population

Table 2. Odds ratio and 95% confidence interval of having menstrual irregularity based on ovarian morphology when compared to the index group on the left

Ovarian morphology	2 PCP n=16	1 PCP n=21	2 GCP n=17	1 GCP n=36
2 PCP n=16	----	9 (CI 0.9 to 84) p<0.05	36 (CI 4 to 351) p=0.0002	75 (CI 8 to 681) p=0.0001
1 PCP n=21	9 (CI 0.9 to 84) p<0.05	----	3 (CI 0.9 to 13) p=0.07	8 (CI 2 to 28) p<0.01
2 GCP n=17	36 (CI 4 to 351) p=0.0002	3 (CI 0.9 to 13) p=0.07	----	2 (CI 0.5 to 8) p=0.29
1 GCP n=36	75 (CI 8 to 681) p=0.0001	8 (CI 2 to 28) p<0.01	2 (CI 0.5 to 8) p=0.29	----

Please note the ---- occurred in boxes because subjects could not be compared to themselves. PCP: Peripheral cystic pattern, GCP: General cystic pattern, CI: Confidence interval, p<0.05 statistically significant

was that of infertile patients undergoing IVM cycles for known PCOM. Unlike in Christ et al.'s study,[16] which used the NIH definition of PCOS, evidence of hyperandrogenism was not used as an inclusion criterion in our study[4,16].

When compared to women with a unilateral PCP ovary, women with bilateral PCP were nine times more likely to experience menstrual irregularities. While there was no difference seen in the rates of menstrual irregularity in women with bilateral GCP ovaries compared to women with a unilateral GCP ovary, a trend was present, which may become statistically significant in a larger study. The presence of a PCP FDP is suspected to occur secondary to a stromal core with increased density and vascular blood flow, which could push the ovarian follicle peripherally. Stromal density and vascular flow have previously been shown to predict the severity of PCOS as they are correlated with levels of ovarian hyperandrogenism[1,17-20]. Unlike PCP ovaries, GCP ovaries do not display increased stromal density and may suggest less androgenic disturbance in the patient, even when both ovaries are found to be multicystic. This would provide a plausible explanation for the difference in menstrual disturbances seen in women with two PCP ovaries, verses one PCP ovary, verses any GCP ovaries.

Study Limitations

Our study has limitations. First, this study is based on the retrospective and subjective assessment of FDP by static, baseline ultrasound images. Although the investigators reviewing the images did not have information regarding the cycle lengths of the subjects and any disagreement between investigators was resolved with a third assessor, we cannot completely rule out the low possibility of a classification bias. Second, our analysis was restricted to a relatively homogenous population of women undergoing infertility assessment and treatment. We cannot determine whether the difference in ovarian morphology represents different, similar syndromes grouped into PCOS or a continuum determined by increased severity of the disease in one population. Anti-Müllerian hormone levels would have been interesting to have; however, they were unavailable as they were not being routinely performed in our clinic at the time the patients were evaluated.

Conclusion

This study affirms the importance of assessing FDP in a population of women with known PCOM and undergoing infertility treatment. A PCP FDP may be useful in identifying a subset of women who are more likely to have worse menstrual disturbances. The mechanism of the relationship between menstrual irregularity and ovarian morphology requires further study to better understand the pathophysiology of this disease.

Ethics

Ethics Committee Approval: Committee for the Protection of Human Subjects approval of the study was obtained.

Informed Consent: Retrospective study.

Peer-review: Internally peer-reviewed.

Authorship Contributions

Concept: G.M., B.G., S.E.E., W.Y.S., M.H.D., Design: G.M., B.G., S.E.E., W.Y.S., M.H.D., Data Collection or Processing: B.G., S.E.E., Analysis or Interpretation: M.H.D., W.Y.S., Literature Search: G.M., B.G., S.E.E., W.Y.S., M.H.D., Writing: G.M.

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Hormonal changes in consecutive clomiphene citrate stimulation cycles and their effect on pregnancy rates

Ardışık klomifen sitrat stimülasyon sikluslarında hormonal değişiklikler ve gebelik oranlarına etkisi

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Abstract

Objective: To determine the relationship between the cumulative effect of sequential clomiphene citrate (CC) treatments in unexplained infertile women with intercycle and intracycle serum hormone changes.

Materials and Methods: Patients who received CC 50 mg in the first cycle (group I, n=34) as ovulation induction and those who received CC 50 mg in the second consecutive cycle (group II, n=18) were compared. Basal (cycle days 2-5) and trigger day (the day that recombinant human chorionic gonadotropin is given) levels of gonadotropin and steroid hormones were measured.

Results: The 17OHP increase on trigger day was found to be statistically significantly higher in group II compared to the basal day (p=0.083). The testosterone (T) response on the trigger day of the patients in group II was found to be statistically significantly higher than that in group I (p=0.023). The number of selected follicles was negatively correlated with a follicle-stimulating hormone decrease and positively correlated with an estradiol increase. Endometrial thickness was positively correlated with a luteinizing hormone increase, and cycle cancelation was positively correlated with decreased estradiol.

Conclusion: Based on this study, it was concluded that the reason for the increased efficiency rate in successive cycles of CC may be the cumulative increase in T and 17OHP levels. However, this result was found not to affect the clinical pregnancy rate.

Keywords: Clomiphene, ovulation induction, steroids

Öz

Amaç: Açıklanamayan infertil kadınlarda, ardışık klomifen sitrat (CC) tedavilerindeki kümülatif etkinin, sikluslar arası ve siklus içi serum hormon değişiklikleri ile arasındaki ilişkiyi belirlemek amaçlandı.

Gereç ve Yöntemler: Ovulasyon indüksiyonu olarak ilk sikluslarında CC 50 mg (grup I, n=34) ve ardarda ikinci siklusta CC 50 mg alan hastaların (grup II, n=18) siklusları karşılaştırıldı. Bazal (siklusun 2-5. günleri) ve ovulasyonun tetiklendiği günlerde (rekombinant insan koryonik gonadotropinin verildiği gün) gonadotropin ve steroid hormon seviyeleri ölçüldü.

Bulgular: Tetik günündeki 17OHP artışı, grup II'de bazal güne göre istatistiksel olarak anlamlı derecede yüksek bulundu (p=0.083). Grup II'deki hastaların tetikleme gününde testosteron (T) yanıtı grup I'dekilere göre istatistiksel olarak anlamlı derecede yüksek bulundu (p=0.023). Seçilen folikül sayısının, folikül uyarıcı hormon azalmasıyla negatif, östradiol artışıyla pozitif korelasyon gösterdiği bulundu. Endometriyal kalınlık ile lüteinize edici hormon artışı arasında, siklus iptali ile östradiol düşüşü arasında pozitif korelasyon bulundu.

Sonuç: Bu çalışmadan yola çıkılarak, CC'nin ardışık sikluslarındaki artan verimin nedeninin, T ve 17OHP seviyelerindeki kümülatif artıştan kaynaklanabileceği sonucuna varılmıştır. Ancak bu sonucun klinik gebelik oranını etkilemediği görüldü.

Anahtar Kelimeler: Klomifen, ovulasyon indüksiyonu, steroidler

PRECIS: We evaluated the cumulative effect of sequential clomiphene citrate treatments in unexplained infertile women, as well as inter- and intra-cycle serum hormone changes.

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Introduction

Ovulation is a physiological process defined by the ruptured release of the dominant follicle capable of fertilization from the ovary into the fallopian tube[1]. The paracrine and autocrine effects of hormones contribute to the regulation of this process[2]. Clomiphene citrate (CC) is a selective estrogen receptor modulator that reduces negative feedback to the hypothalamus by competing with estrogen for binding to the hypothalamic estrogen receptors. It is the first-line agent of choice to support ovulation for treating infertility in unexplained infertile women. It stimulates the secretion of hypothalamic gonadotropin-releasing hormone and then activates ovarian stimulation by increasing the release of gonadotropins[3,4]. It seems possible that CC stimulation can alter the endocrine environment both systemically and locally in the ovary. However, most women will achieve pregnancy by going through more than one cycle of CC stimulation. Because the cumulative effect of CC is mentioned.
It is not exactly known how the serum hormone levels change in the next cycle following a CC stimulation cycle that does not result in pregnancy even though there is follicle development. Until date, many studies have been conducted to examine the effects of gonadotropins and steroid hormones on folliculogenesis in infertility treatments. However, most of the studies were performed either in patients with polycystic ovary syndrome (PCOS) or in assisted reproductive technology (ART) treatments.
This study aims to compare the first cycle of CC used for ovulation stimulation and the second consecutive CC cycle (immediately after) in unexplained infertile women, and to examine the effect on endogenous hormones, through which hormones the cumulative activity develops and whether this affects pregnancy rates.

Materials and Methods

This study was conducted between August 2019 and March 2020 in a tertiary referral hospital infertility outpatient clinic as a prospective case-control study. Ethical approval was obtained from the Local Ethics Committee of University of Health Sciences Turkey, İstanbul Bağcılar Training and Research Hospital (approval number: 2019.08.1.04.061) for the study of "hormonal changes in incremental CC stimulation doses, and their effect on pregnancy rates". This study was conducted as a subgroup analysis of the other. The study was conducted following the Declaration of Helsinki and its later amendments. All participants were included in the study after obtaining informed consent.
Fifty-two CC cycles of 34 unexplained infertile women were included in the study. The patients given CC 50 mg first cycle were divided into group I (n=34), and the patients given CC 50 mg for the second consecutive month as group II (n=18). Of the 34 patients in group I, 5 conceived. CC 100 mg was given in the next cycle because six patients did not develop follicles with CC 50 mg. Five patients wanted to interrupt the treatment. Eighteen patients who ovulated with CC 50 mg were taken into the second cycle. Women between the ages of 20 and 35 who were hormonally eugonadotropic and had no Müllerian anomaly or bilateral tubal obstruction on hysterosalpingography were included in the study. Serum Anti-Müllerian hormone (AMH) levels, body mass index (BMI), homeostatic model assessment for insulin resistance (HOMA-IR) and the type of sterility (primary/secondary) were recorded. Couples with normal spermiograms or mild male factor infertility (i.e. male partners with only one of the following abnormalities: Sperm counts <20 million/mL, normal morphology <4% or sperm motility <40% and post-wash total motile sperm counts ≥5 million/mL) of their male partners were included in the study. The following exclusion criteria were applied: severe male factor, recurrent pregnancy loss, use of co-medication (myoinositol, metformin, cortisone/prednisolone), tubal obstruction, additional endocrine (such as PCOS) or medical disorders, women with AMH <1.1 ng/mL, previous pelvic surgery, ovarian cysts, endometriosis, those with a BMI >30 kg/m^2. Those who became pregnant with the first cycle CC or those whose follicle development could not be achieved with CC 50 mg were excluded from the second cycle CC 50 mg group.
Folliculometry with transvaginal sonography and pre-stimulation hormonal testing [follicle stimulating hormone (FSH), luteinizing hormone (LH), progesterone (P_4), estradiol (E_2), prolactin (PRL), thyroid-stimulating hormone (TSH), AMH, androstenedione (A_4), total testosterone (T), dehydroepiandrosterone sulfate (DHEA-S), 17 hydroxyprogesterone (17OHP)] were performed on the second to fifth day (basal day). Blood sampling from the antecubital vein was performed after an overnight fasting between 8:00-10:00 am. CC (Klomen 50 mg; Kocak Farma-Turkey) stimulation was begun with an initial dose of 50 mg/day from the fifth day to the ninth day of the menstrual cycle having early cycle blood P_4 <0.5 ng/mL and E_2 <50 pg/mL levels. Beginning with the 11th day, patients underwent every other day transvaginal sonografic monitoring of endometrial thickening and follicular growth. When the leading follicle reached a mean diameter of 18-20 mm, ovulation was induced by subcutan application of choriogonadotropin alfa (rHCG, Ovitrelle 250 μgr, Merck Serono-Turkey) (trigger day). All hormonal tests (FSH, LH, E_2, P_4, A_4, T, DHEA-S, 17OHP) were performed again before rHCG application.
Intrauterine insemination or timing intercourse was performed within the 36th to 40th hours. Blood betaHCG levels were measured on the 15th- to 20th-day postinsemination. If the beta-HCG blood level was higher than 20 mIU/mL, the conception was confirmed. Cycle cancellations were due to no ovarian response for 21 days follow up or excessive ovarian responses (blood E_2 level >1500 pg/mL or more than two selected follicles ≥16 mm, on the trigger day). Patients who ovulated with CC 50 mg but did not become pregnant were given CC 50 mg in the same manner for the second consecutive cycle with the next menstrual cycle. For women who could not achieve ovulation

with CC 50 mg, CC 100 mg treatment was started in the next cycle. However, this group was excluded in the study.

Primary outcome measure: Changes in the endogenous blood levels of FSH, LH, E_2, P_4, A_4, T, DHEA-S, and 17OHP in CC cycles.

Secondary outcome measure: Clinical pregnancy rate (CPR), live birth rate (LBR).

All hormones were measured in the biochemistry laboratory of our hospital using the UniCel DxI 800 immunochemistry analyzer (Beckman Coulter Inc., USA) according to the manufacturer's assay instructions and requirements. Access FSH, LH, PRL and TSH values were assayed using a two-site immunoenzymatic (sandwich) method. Access E_2, T, P_4, A_4, DHEA-S, and 17OHP values were assayed using the competitive immunoenzymatic method. Basal AMH concentrations were assayed following a highly specific enzyme-linked immunosorbent method. The coefficients of variation intraassay and interassay tests of these hormones are as follows [mean ± standard deviation (SD)]: 7.33±1.54 mIU/mL for FSH, 5.89±2.84 mIU/mL for LH, 40.1±16.5 pg/mL for E_2, 0.59±0.34 ng/mL for P_4, 1.04±0.44 ng/mL for A_4, 200.76±74.5 μg/dL for DHEA-S, 0.48±0.18 ng/mL for T, 1.84±0.59 ng/mL for 17OHP, 4.86±3.35 ng/mL for AMH.

Statistical Analysis

Mean SD, median, minimum, and maximum values are given in the descriptive statistics for continuous data. Number percentages are given for discrete data. The Shapiro-Wilk test and Stem-and-Leaf Plot test were used to examine the compatibility of continuous data to the normal distribution. In the comparison of continuous variables between independent groups, Student's t-test was used for normally distributed data and Mann-Whitney U test was used for data not normally distributed. In dependent groups, the t-test (paired sample t-test) was used for data conforming to the normal distribution. The Wilcoxon test was used for the data that did not show a normal distribution. Fisher's Exact and chi-square tests were used in group comparisons (cross tables) of nominal variables. The analyzes to be used were decided by testing the data for normal distribution, which is the most important assumption in the analyses. The IBM SPSS Statistics 20 program was used for evaluations and a p-value <0.05 was accepted to be statistically significant.

Results

The mean age of the women included in the study was 26.62±3.54 (20-33). Twenty-four women (70%) were primary infertile, and 10 women (30%) were secondary infertile (total n=34). Demographic characteristics of the groups are given in Table 1. The cycles were divided into 2 groups as the first cycle CC (n=34) (group I) and consecutive second cycle CC (n=18) (group II). Women in both groups were similar in terms of mean age, AMH (1.1-17.1), HOMA-IR (0.66-5.03), BMI (17.1-38.6), partner's spermiogram values, number of follicles selected after treatment (0-4) and cycle cancelation rates (p>0.05).

The comparison of basal day and trigger day hormonal values between groups is given in Table 2 and the variation of the differences in hormone values is given in Table 3. The correlation between the change (difference) hormones and the number of follicles selected is given in Table 4.

There were 5/34 (14.7%) and 0/18 (0%) conceptions in groups I and II, respectively. In other words, clinical pregnancy occurred in 5 of 52 cycles (CPR: 9.6%). One of them ended in miscarriage at 6 weeks. Four had live births at term (LBR: 7.7%). A comparison of hormonal change values of women with and without clinical pregnancy is given in Table 5.

Discussion

In this study, it was found that only the increase in T levels in consecutive cycles of CC was statistically significantly higher, but this did not affect the CPR. Several markers have been introduced for the cycle outcome prediction. However, most studies have been conducted either CC in women with PCOS or with gonadotropins in ART cycles. No study could be found in the literature regarding the clinical significance of hormonal change created by consecutive CC cycles in patients with normal ovarian reserves.

Table 1. Demographic characteristics of the groups

	Group I (n=34) Mean ± SD Median (min-max)	Group II (n=18) Mean ± SD Median (min-max)	Test statistics	p-value
Age (year)	27.18±3.65 26 (20-33)	25.56±3.17 25 (20-33)	t=1.593	[a]0.117
AMH (ng/mL)	4.51±2.80 3.46 (1.12-11.42)	4.93±3.80 3.36 (1.46-17.10)	U=300.5	[b]0.916
HOMA-IR	1.94±0.92 1.74 (0.72-5.03)	1.92±0.99 1.60 (0.66-4.22)	U=294.5	[b]0.825
BMI (kg/m^2)	26.40±5.04 26.28 (17.1-38.6)	25.67±4.60 25.28 (17.1-36)	t=0.509	[a]0.613

[a]: Student's t-test, [b]: Mann-Whitney U test, SD: Standard deviation, Min: Minimum, Max: Maximum, AMH: Anti-Müllerian hormone, HOMA-IR: Homeostatic model assessment for insulin resistance, BMI: Body mass index

Table 2. Comparison of basal day and trigger day hormone values of the groups

		Basal day Mean ± SD Median (min-max)	Trigger day Mean ± SD Median (min-max)	Test statistics	p-value
FSH (mIU/mL)	Group I	7.57±1.87 7.24 (4.17-12.58)	6.46±2.97 5.8 (3.49-16.68)	Z=-2.269	[c]**0.023***
	Group II	6.79±1.53 7.01 (4.15-9.12)	6.12±2.01 5.48 (3.51-11.50)	Z=-1.241	[c]0.215
	Test statistics	t=1.500	U=284.5		
	p-value	[a]0.140	[b]0.805		
LH (mIU/mL)	Group I	6.16±3.22 5.43 (2.22-13.63)	16.53±13.59 10.42 (4.33-55.61)	Z=-4.976	[c]**0.000***
	Group II	5.87±3.26 4.99 (0.70-11.99)	17.92±16.46 10.80 (5.10-60.98)	Z=-3.550	[c]**0.000***
	Test statistics	U=284.5	U=296.0		
	p-value	[b]0.805	[b]0.984		
E_2 (pg/mL)	Group I	39.62±15,73 36 (19.10-84.0)	371.82±322.42 320 (40.8-1166.0)	Z=-4.994	[c]**0.000***
	Group II	38.39±14.58 37.3 (4.3-60.7)	370.64±293.35 267.2 (37.3-1024.0)	Z=-3.680	[c]**0.000***
	Test statistics	U=281.0	U=284.0		
	p-value	[b]0.752	[b]0.798		
P_4 (ng/mL)	Group I	0.62±0.44 0.58 (0.05-1.82)	1.25±0.87 1.04 (0.27-3.70)	Z=-3.895	[c]**0.000***
	Group II	0.69±0.48 0.53 (0.16-1.91)	1.33±1.05 0.91 (0.22-4.60)	Z=-2.853	[c]**0.004***
	Test statistics	U=270.0	U=295.5		
	p-value	[b]0.595	[b]0.976		
A_4 (ng/mL)	Group I	1.02±0.48 0.92 (0.39-2.11)	1.67±0.54 1.55 (0.82-2.88)	t=-6.324	[a]**0.000***
	Group II	1.07±0.42 1.08 (0.46-2.11)	1.76±0.74 1.71 (0.51-3.36)	t=-4.499	[a]**0.000***
	Test statistics	t=-0.383	t=-0.908		
	p-value	[a]0.704	[a]0.369		
DHEA-S (µ g/dL)	Group I	190.12±66.06 175.8 (110-361.2)	212.14±79.45 204.8 (130.5-445.8)	Z=-2.486	[c]**0.013***
	Group II	215.78±85.03 192.1 (91.2-357.6)	255.82±114.65 230.5 (143.8-600.5)	Z=-2.107	[c]**0.035***
	Test statistics	U=182.5	U=162.0		
	p-value	[b]0.442	[b]0.104		
T (ng/mL)	Group I	0.46±0.19 0.42 (0.13-0.98)	0.55±0.21 0.52 (0.32-1.10)	t=-2.422	[a]**0.024***
	Group II	0.51±0.19 0.45 (0.23-0.88)	0.71±0.25 0.62 (0.32-1.14)	t=-4.749	[a]**0.000***
	Test statistics	t=-0.896	t=-2.361		
	p-value	[a]0.376	[a]**0.023***		

Table 2. Continued

		Basal day Mean ± SD Median (min-max)	Trigger day Mean ± SD Median (min-max)	Test statistics	p-value
17OHP (ng/mL)	Group I	1.73±0.58 1.8 (0.86-2.65)	2.23±1.16 2.36 (0-3.89)	Z=-0.889	[c]0.374
	Group II	1.98±0.64 2.17 (0.89-2.88)	3.48±1.49 3.86 (1.98-6.20)	Z=-2.366	[c]0.018*
	Test statistics	U=37.5	U=21.0	-	-
	p-value	[b]0.600	[b]0.083	-	-
Number of follicles selected	Group I	-	1.50±0.99 1 (0-4)	-	-
	Group II	-	1.56±0.78 2 (0-3)	-	-
	Test statistics	-	U=283.5	-	-
	p-value	-	[b]0.646	-	-
Male factor n (%)	Group I (mild) (absent)	-	3 (8.8%) 31 (91.2%)	-	-
	Group II (mild) (absent)	-	2 (11.1%) 16 (88.9%)	-	-
	Test statistics	-	χ^2 =0.071	-	-
	p-value	-	[d]1.000	-	-
Cycle cancelation n (%)	Group I (yes) (no)	-	6 (17.6%) 28 (82.4%)	-	-
	Group II (yes) (no)	-	3 (16.7%) 15 (83.3%)	-	-
	Test statistics	-	χ^2=0.008	-	-
	p-value	-	[d]1.000	-	-

[a]: Student's t-test, [b]: Mann-Whitney U test, [c]: Wilcoxon test, [d]: Chi-square, SD: Standard deviation, Min: Minimum, Max: Maximum, *p<0.05, FSH: Follicle stimulating hormone, LH: Luteinizing hormone, E_2: Estradiol, P_4: Progesterone, A_4: Androstenedione, DHEAS: Dehydroepiandrosterone sulfate, T: Total testosterone, 17OHP: 17 hydroxyprogesterone

In CC cycles, it has been shown that FSH increases with the first dose of CC and starts to decrease in the middle of the cycle with the last dose[5]. In our study, we found that FSH decreased statistically significantly in group I on the trigger day compared with the basal day. We also found a decrease in consecutive second cycle CC patients, but the difference was not statistically significant. Perhaps the cumulative effect of CC lasting up to 6 weeks can be explained by this effect on FSH[6]. Since we did not sample in the mid-follicular phase, we could not catch the physiological FSH peak[7]. As in the natural menstrual cycle[2,5-9], in this study, all hormones (LH, E_2, P_4, A_4, DHEA-S, and T) except FSH increased significantly on the trigger day according to the basal day values in both groups. However, when the two groups were compared with each other, the FSH change values (decrease amounts), LH, E_2, A_4, P_4 DHEA-S, T, and 17OHP change values (increase amounts) were found to be similar from basal day to trigger day. This result does not support the cumulative effect of the CC.

Previously, androgens were suspected to cause follicular atresia[10,11]. However, in subsequent studies, androgens have been shown to play an important role in follicular development[12-14]. Even today, numerous studies on androgens continue to produce conflicting results. The major androgens in the serum of normal cycling women are A_4, DHEA-S, T, and dihydrotestosterone (DHT)[15,16]. 17OHP is synthesized from P_4 with 17αhydroxylase and converted to A_4, T, E_2 by aromatase in the adrenal gland[17]. In this study, T levels were significantly increased in group II compared with group I. It was therefore concluded that this situation is one of the main responsible factors of CC in follicular development. Fanelli et al.[18] showed that the upper levels of 17OHP and T were higher in the luteal phase than in the follicular phase, but androgen levels did not change during the menstrual cycle. Studies have suggested that cumulative A_4 response[19], basal DHEA-S[20] or basal T levels[21,22] are predictors of follicle number, fertilized oocyte, mature oocyte count, embryo development in ART cycles. In contrast, Abide Yayla et al.[23] showed that basal

Table 3. Comparison of the hormone values on the trigger day of the groups according to basal day hormone values (differences)

	Group I Mean ± SD Median (min-max)	Group II Mean ± SD Median (min-max)	Test statistics	p-value
FSH change (difference) on trigger day relative to basal day	-1.10±3.77 -1.55 (-8.78-10.51)	-0.67±2.60 -1.14 (-4.38-4.0)	U=258.0	[a]0.442
LH change (difference) on trigger day relative to basal day	10.37±13.08 5.12 (-0.15-47.63)	12.05±15.21 6.03 (-2.82-50.52)	U=271.0	[a]0.608
E_2 change (difference) on trigger day relative to basal day	332.20±315.23 243.1 (-0.6-1082)	332.26±290.48 240.5 (-4.7-983.4)	U=281.0	[a]0.752
A_4 change (difference) on trigger day relative to basal day	0.62±0.47 0.64 (-0.52-1.40)	0.69±0.63 0.75 (-0.26-2.16)	U=185.5	[a]0.787
DHEA-S change (difference) on trigger day relative to basal day	22.02±36.78 18.35 (-46.2 (94.30)	40.03±76.99 34.1 (-61.4-279.60)	U=183.5	[a]0.587
T change (difference) on trigger day relative to basal day	0.09±0.18 0.08 (-0.49-0.45)	0.19±0.17 0.18 (017-0.53)	U=133.0	[a]0.060
17OHP change (difference) on trigger day relative to basal day	0.49±1.29 0.91 (-1.73-1.92)	1.49±1.22 1.31 (0.41-4.03)	U=21.0	[a]0.299

[a]: Mann-Whitney U test, SD: Standard deviation, Min: Minimum, Max: Maximum, FSH: Follicle stimulating hormone, LH: Luteinizing hormone, E_2: Estradiol, A_4: Androstenedione, DHEAS: Dehydroepiandrosterone sulfate, T: Total testosterone, 17OHP: 17 hydroxyprogesterone

Table 4. Correlation between the change (difference) in hormones and the number of follicles selected

	Number of selected follicles	
	r	p
FSH change (difference) on trigger day relative to basal day	-0.424	**0.002***
LH change (difference) on trigger day relative to basal day	-0.111	0.439
E_2 change (difference) on trigger day relative to basal day	0.642	**0.000***
A_4 change (difference) on trigger day relative to basal day	0.030	0.854
DHEA-S change (difference) on trigger day relative to basal day	-0.035	0.829
T change (difference) on trigger day relative to basal day	-0.175	0.273
17OHP change (difference) on trigger day relative to basal day	-0.244	0.362

r: Correlation coefficient, *p<0.05, FSH: Follicle stimulating hormone, LH: Luteinizing hormone, E2: Estradiol, A4: Androstenedione, DHEAS: Dehydroepiandrosterone sulfate, T: Total testosterone, 17OHP: 17 hydroxyprogesterone

serum DHEA-S and T levels have no value in predicting any cycle outcome parameter in a study of 120 women with diminished ovarian reserve. In this study, although hormonally T levels were significantly higher, and there was no difference between A_4, 17OHP, and DHEA-S levels, we could not find a relationship between them and the number of selected follicles and pregnancy outcomes.

It was found that as the decrease in FSH on the trigger day and the increase in E_2 increased, the number of selected follicles increased. When the relationship of hormonal changes between the groups with cycle cancelation was examined, no relationship was found with any hormonal change, except for the E_2 response.

Regardless of the groups, no difference was found between the hormonal changes of women with or without conception. This result may have occurred because there were no patients with low ovarian reserve or PCOS in the study group. In CC cycles, pregnancy rates per cycle are reported to be 9.7-24.6% in anovulatory women[24] and 11.4-21.5% in ovulatory women[25]. Our results were consistent with this, and 5 women (9.6%) remained pregnant. However, all the pregnant women were from group I. We concluded that the main reason for this was the small number of patients in the groups.

Study Limitations

Owing to the rapid development in ART treatments, studies on first-line infertility treatments have unfortunately lost their appeal. The study strength is that it draws attention to this issue again. The small number of patients in the groups and the absence of a control group are the limitations of the study.

Table 5. Comparison of hormonal change values of women with and without clinical pregnancy

	Woman with clinical pregnancy Mean ± SD Median (min-max)	Woman without clinical pregnancy Mean ± SD Median (min-max)	Test statistics	p-value
FSH change (difference) on trigger day relative to basal day	0.70±6.4 1.06 (-8.78-7.64)	-1.13±2.99 -1.62 (-5.42-10.51)	U=75.0	[a]0.218
LH change (difference) on trigger day relative to basal day	15.45±13.75 10.57 (2.57-34.19)	10.47±13.80 5.47 (-2.82-50.52)	U=87.0	[a]0.395
E2 change (difference) on trigger day relative to basal day	229.76±148.23 243.1 (59.8-385.3)	343.36±315.11 243.9 (-4.70-1082)	U=102.0	[a]0.701
A4 change (difference) on trigger day relative to basal day	0.70±0.08 0.70 (0.64-0.77)	0.65±0.55 0.68 (-0.52-2.16)	U=35.5	[a]0.877
DHEA-S change (difference) on trigger day relative to basal day	14.75±1.48 14.75 (13.7-15.8)	30.24±58.21 21.21 (-61.6-279.6)	U=30.0	[a]0.624
T change (difference) on trigger day relative to basal day	0.05±0.05 0.05 (0.02-0.09)	0.14±0.19 0.12 (-0.49-0.53)	U=23.0	[a]0.380

[a]: Mann-Whitney U, SD: Standard deviation, Min: Minimum, Max: Maximum, FSH: Follicle stimulating hormone, LH: Luteinizing hormone, E_2: Estradiol, A_4: Androstenedione, DHEAS: Dehydroepiandrosterone sulfate, T: Total testosterone, 17OHP: 17 hydroxyprogesterone

Conclusion

To our knowledge, this is the first study to examine hormonal changes after consecutive CC stimulation cycles in unexplained infertile women with normal ovarian reserve. The findings of this clinical study suggest that CC had a cumulative effect on T. However, the observed effects of CC stimulation on the hormonal profile seemed to be of minor clinical relevance. This still needs to be further studied in covering the luteal phase and larger prospective studies.

Ethics

Ethics Committee Approval: Ethical approval was obtained from the Local Ethics Committee of University of Health Sciences Turkey, İstanbul Bağcılar Training and Research Hospital (approval number: 2019.08.1.04.061).
Informed Consent: All participants were included in the study after obtaining informed consent.
Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: E.E.K., Ö.K.A., T.K., Concept: E.E.K., E.Ş.Ö., Design: E.E.K., E.Ş.Ö., Data Collection or Processing: E.E.K., Ö.K.A., T.K., Analysis or Interpretation: E.E.K., E.Ş.Ö., T.K., Literature Search: E.E.K., Ö.K.A., Writing: E.E.K., E.Ş.Ö.
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The role of inflammation, oxidation and Cystatin-C in the pathophysiology of polycystic ovary syndrome

Polikistik over sendromunun patofizyolojisinde enflamasyon, oksidasyon ve Sistatin-C'nin rolü

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Abstract

Objective: The relationship between Cystatin-C levels and inflammatory, oxidant, and antioxidant markers in polycystic ovary syndrome (PCOS) was investigated.

Materials and Methods: A total of 96 participants were included in the study as PCOS (n=58) and control (n=38) groups. Tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1B), interleukin 6 (IL-6), malondialdehyde (MDA), superoxide dismutase (SOD), and Cystatin-C were evaluated by ELISA method. Relationships metabolic and endocrine parameters seen in PCOS were examined. Univariate and multivariate logistic regression analyzes were performed to identify risk factors that may affect the PCOS group. Bivariate correlations were investigated by the Spearman's correlation analysis.

Results: While Cystatin-c, TNF-α, IL-1B, IL-6, MDA were found to be higher in patients with PCOS compared with the control group, SOD was found to be lower than the control group ($p<0.05$). In the correlation analysis, increased Cystatin-C levels were found to be associated with high IL-6 ($r=0.214$, $p=0.037$) and low SOD levels ($r=-0.280$, $p=0.006$).

Conclusion: In our study, it was found that the increase in Cystatin-C levels was associated with an increase in IL-6 and a decrease in SOD. These results may bring up different treatment options to reduce cardiovascular risks for treating PCOS.

Keywords: Polycystic ovary syndrome, Cystatin-C, interleukin, oxidation, superoxide dismutase

Öz

Amaç: Polikistik over sendromunda (PKOS) Sistatin-C düzeyleri ile enflamatuvar, oksidan ve antioksidan belirteçler arasındaki ilişkinin araştırılması amaçlandı.

Gereç ve Yöntemler: Çalışmaya PKOS (n=58) ve kontrol (n=38) grubu olarak toplam 96 katılımcı dahil edildi. Tümör nekroz faktör-alfa (TNF-α), interlökin-1 beta (IL-1B), interlökin 6 (IL-6), malondialdehit (MDA), süperoksit dismutaz (SOD) ve Sistatin-C markerları ELISA yöntemi ile değerlendirildi. PKOS grubunu etkileyebilecek olası risk faktörlerini belirlemek için tek değişkenli ve çok değişkenli lojistik regresyon analizi yapıldı. İki değişkenli korelasyonlar Spearman korelasyon analizi ile araştırıldı.

Bulgular: PKOS hastalarında Sistatin-C, TNF-α, IL-1B, IL-6, MDA, kontrol grubuna göre daha anlamlı olarak yüksek bulunurken, kontrol grubunda SOD daha yüksek bulundu ($p<0,05$). Korelasyon analizinde, artan Sistatin-C seviyeleri, yüksek IL-6 ($r=0,214$, $p=0,037$) ve düşük SOD seviyeleri ($r=-0,280$, $p=0,006$) ile ilişkili bulundu.

Sonuç: Çalışmamızda kardiyovasküler hastalık belirteci olan Sistatin-C düzeylerindeki artışın yüksek IL-6 ve düşük SOD seviyeleri ile ilişkili olduğu bulundu. Bu sonuçlar PKOS tedavisinde kardiyovasküler riskleri azaltmak için farklı tedavi seçeneklerini gündeme getirebilir.

Anahtar Kelimeler: Polikistik over sendromu, Sistatin-C, interlökin, oksidasyon, süperoksit dismutaz

PRECIS: This study is a case-control study evaluating the relationship between Cystatin-C levels and inflammatory, oxidant, and antioxidant markers in polycystic ovary syndrome.

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Introduction

Polycystic ovary syndrome (PCOS) is a disease with clinical or laboratory findings of hyperandrogenism, polycystic ovary appearance, and menstrual irregularity. It is often observed in women of reproductive age[1]. The international prevalence rate ranges from 5 to 21%[2]. Although the etiology is not clearly known, disruption of oxidant mechanisms and increased inflammatory mediators are thought to be the cause[1,3,4]. Studies have shown that, depending on the increase in adipose, inflammatory mediators such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6) and malinaldehyde (MDA) levels increase, while superoxide dismutase (SOD) decreases[5,6]. As a result, the deterioration in oxidant-antioxidant balance and increase in inflammatory markers are observed due to increased adipose tissue and hyperandrogenemia. This situation increases diseases that increase cardiovascular risk, such as insulin resistance, obesity, dyslipidemia, and type 2 diabetes mellitus[4].

Cystatin-C is an extracellular cysteine protease inhibitor. It is a low molecular weight cationic protein[7]. It is a strong predictor of not only renal failure but also all-cause mortality, such as cardiovascular disease and diabetes mellitus[8]. It has also been significantly associated with asymptomatic coronary artery disease in patients with metabolic syndrome with normal renal function[9]. Because of these data, Cystatin-C was examined in studies due to the increased cardiovascular risk in polycystic ovarian disease, and this marker was found to be statistically significantly higher in the PCOS group than in the healthy group[10]. In previous studies, either inflammation and oxidative-antioxidative markers or markers such as Cystatin-C were studied. In this study, it was stated that high Cystatin-C levels in patients with PCOS were important in identifying patients at cardiovascular risk[11].

However, it is unclear whether the increase in Cystatin-C is due to increased inflammation or the deterioration of oxidant-antioxidant mechanisms. This study investigated the relationship between Cystatin-C elevation and inflammatory and oxidant-antioxidant mediators. If it is associated with these mechanisms, targeted therapy may come to the fore in terms of cardiovascular protection.

Materials and Methods

Study Design and Participants

Patients over the age of 18 who applied to Yozgat Bozok University Medical Faculty Hospital between 01.01.2022 and 01.04.2022 were included in the study. The Yozgat Bozok University Local Ethical Committee approved the present study (2017-KAEK-189_2021.12.29_02) and informed consent was obtained from all participants.

The diagnosis of PCOS was made according to the Rotterdam criteria. These criteria were clinical and/or biochemical hyperandrogenemia, presence of oligomenorrhea (interval between two menstrual periods more than 35 days) or amenorrhea (no vaginal bleeding for at least six months), and ultrasonographic polycystic ovary appearance (≥12 follicles measuring 2-9 mm in diameter, or ovarian volume >10 mL in at least one ovary)[12]. The presence of acne and/or hirsutism and/or alopecia were evaluated as clinical signs of hyperandrogenemia. Feriman Galway's scoring was used for hirsutism. Nine different parts of the body, upper lip, chin, chest, upper back, lower back, upper abdomen, lower abdomen, arm, and thigh, were scored between 1 and 4 and a total score of 8 and above was considered hirsutism[13]. Findings of hyperandrogenemia and menstrual patterns were recorded in the database at the first diagnosis. The demographic and laboratory data of the patients were recorded retrospectively from the hospital database. Demographic features included waist to hip ratio (WHR), body mass index (BMI), gravidity, parity, and abortion. The parameters evaluated in the study were examined from the blood samples collected for diagnostic purposes before the treatment was initiated.

Exclusion criteria from the study were the presence of chronic systemic disease, infectious and inflammatory diseases, hormone replacement therapy, use of oral contraceptives or drugs for insulin resistance, patients under 18 years of age, presence of psychiatric disorder and drug use for it, history of bariatric surgery, thyroid dysfunction. Secondary causes of clinical and/or biochemical hirsutism and oligomenorrhea, such as congenital adrenal hyperplasia, androgen-secreting tumors, Cushing's syndrome, hyperprolactinemia, thyroid dysfunction, and adrenal disorders were excluded.

Anthropometric Measurements

A weight measurement of the patients was made with a digital scale, with at least clothes and no shoes. Height measurements were made while standing without shoes. BMI was obtained by dividing weight in kilograms (kg) by height (m^2) (kg/m^2). Determined according to BMI World Health Organization's criteria. WHR; was obtained by dividing the waist circumference measured at the thinnest point between the rib and the iliac crest with the hip circumference measured from the widest part of the hips.

Ultrasonography Assessment

Gynecological ultrasound was performed on the second or third day of menstruation with a 7.5 MHz transvaginal transducer or a 5 MHz transabdominal transducer. Antral follicles were measured in three dimensions, and those with an average diameter of 2-9 mm were counted.

Biochemical Measurements

All blood samples used in the study were taken between 08.00 and 09.00 in the morning in the early follicular phase on the second or third day of the menstrual cycle. Pituitary, adrenal and gonadal axis hormones were checked in all patients due to amenorrhea and hirsutism complaints. Liver and kidney

function tests, hemogram, serum lipid levels, fasting plasma glucose, and fasting insulin levels were measured. Serum follicle-stimulating hormone, luteinizing hormone (LH), prolactin, insulin, and thyroid-stimulating hormone (TSH) levels were determined by chemiluminescent immunometric assays using a Cobas 6000 analyzer (Roche, Swiss) method. Fasting glucose, total cholesterol, high-density lipoprotein cholesterol and triglyceride levels (TG) were measured spectrophotometrically using an enzymatic colorimetric assay (Roche Integrated system, Mannheim, Germany). Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula. Insulin resistance was calculated using the homeostatic model assessment for the insulin resistance index (HOMA-IR). The HOMA-IR formula is fasting plasma glucose (mg/dL) x fasting serum insulin (mU/mL)/405[14].

Blood samples were collected from each patient after a 12-hour fasting period for TNF-α, interleukin- 1 beta (IL-1β), and IL-6. Whole blood samples were centrifuged for 10 min at 4000 rpm, and the supernatants were kept at -80 °C until the assays were performed by an investigator who was blind to each patient's status. Commercial enzyme-linked immunosorbent assay (ELISA) kits were used for measuring Cystatin-C, TNF-α, IL-1β, and IL-6 (Bioassay technologies, China) levels using appropriate wavelengths on a microplate reader (BioTek Instruments, EL x 800 TM, USA) following the assay instructions. Concentrations were calculated over the standard curves. Serum MDA level was determined according to Göçmen et al.[15] Total SOD activity was examined using the SOD Activity Assay kit (Rel Assay Diagnostics kit; Mega Tıp, Gaziantep, Turkey), according to the manufacturer's instructions.

Statistical Analysis

The statistical package program SPSS 20 (IBM Corp. released 2011. IBM SPSS Statistics for Windows, version 20.0, Armonk, NY: IBM Corp.) was used to evaluate the data. Data were expressed as mean ± standard deviation and in percentages. Continuous variables were investigated using analytical methods (Kolmogorov-Simirnov/Shapiro-Wilk's test) to determine whether they were normally distributed. The Mann-Whitney U test was used for the non-parametric numerical data, while the Student's t-test was adopted for the parametric numerical data. Relationships between categorical variables were analyzed by the chi-square test. Bivariate correlations were investigated by the Spearman's correlation analysis. Univariate and multivariate logistic regression analyzes were performed to identify risk factors that may affect the PCOS group. P<0.05 were accepted as statistically significant.

Results

A total of 96 patients were included in the study, 60.4% of whom were PCOS (n=58) and 39.6% were from the control group (n=38). When the demographic data of both groups were analyzed, gravida and parity were found to be significantly lower in the PCOS group (p<0.05) (Table 1). When the laboratory data of the patients were evaluated, it was observed that the TSH level was statistically significantly lower in the PCOS group (p<0.05). There was no significant difference between the two groups in fasting glucose, fasting insulin, and cholesterol levels, which are cardiovascular risk markers, but Cystatin-C level was found to be high in the PCOS group (p<0.05) (Table 2). When the inflammatory, oxidant, and antioxidant markers of both groups were compared, it was seen that IL-1β, IL-6, TNF-α, and MDA were statistically significantly higher and SOD was low in patients with PCOS (p<0.05) (Table 2).

In the multivariate regression analysis, TNF-α [odds ratio (OR)=1.2, 95% confidence interval (CI)=1.1-1.3], IL-1β (OR=1.1, 95% CI=1.1-1.3), IL-6 (OR=3.9, 95% CI=1.1-13.5) and Cystatin-C (OR=11.7, 95% CI=2.8-98.1) levels were found to be independently high in the PCOS group (Table 3).

When the relationship between Cystatin-C elevation and these markers was evaluated (in the bivariate correlation), it was observed that the increase in Cystatin-C was associated with an increase in IL-6 levels (r=0.214, p=0.037) and a decrease in SOD levels (r=-0.280, p=0.006) (Table 4).

Discussion

This study showed that IL-1β, IL-6, TNF-α, and MDA were significantly higher and SOD was low in patients with PCOS. Again in the study, Cystatin-C, which is a risk factor for cardiovascular diseases, was found to be high in the PCOS group. When the relationship between the elevation

Table 1. Demographic data of PCOS and control group

	Control	PCOS	OR	95% CI		p
Age (years)	30.2±5.2	28.2±4.0	1.998	0.090	3.906	0.075
BMI (kg/m²)	5.9±0.9	6.1±1.3	-0.178	-0.715	0.359	0.851
WHR	0.8±0.1	0.8±0.1	-0.030	-0.061	0.001	0.075
Gravidity	1.8±1.3	0.6±0.9	1.281	0.698	1.865	<0.001
Parity	1.4±1	0.4±0.7	0.988	0.556	1.420	<0.001
Abortion	0.3±0.9	0.1±0.4	0.192	-0.149	0.532	0.391

Data are shown as mean ± SD. BMI: Body mass index, WHR: Waist circumference hip circumference ratio, PCOS: Polycystic ovary syndrome, OR: Odds ratio, SD: Standard deviation, CI: Confidence interval

Table 2. Comparison of biochemical characteristics between the two groups

	Control	PCOS	OR	95% CI		p
Fasting glucose (mg/dL)	93.7±43	88.9±8.1	4.862	-7.186	16.909	0.301
Fasting insulin (µIU/mL)	10.3±5.2	13.7±20.9	-3.395	-11.136	4.347	0.314
FSH (IU/L)	5.7±1.6	5±1.5	0.772	-0.483	2.027	0.180
LH (IU/L)	5±2.4	8.8±15.7	-3.795	-15.819	8.228	0.278
E2 (IU/L)	36.3±19.5	45.1±22.6	-8.869	-27.015	9.278	0.233
TSH (mIU/L)	2.7±1.5	2.0±0.7	0.765	0.272	1.257	0.015
LDL (mg/dL)	94.3±35.1	101.3±25	-6.966	-20.303	6.372	0.366
HDL (mg/dL)	52.9±11.8	56.4±14.5	-3.521	-9.722	2.680	0.242
Cholesterol (mg/dL)	169.8±36.4	175.3±27.6	-5.498	-19.669	8.672	0.416
Triglyceride (mg/dL)	93.4±49.4	97.7±52.4	-4.325	-27.839	19.189	0.758
TNF-α (pg/mL)	27±7.2	36.4±10.0	-9.366	-13.103	-5.629	<0.001
IL-1β (pg/mL)	35.1±8.6	47.6±9.9	-12.431	-16.334	-8.528	<0.001
IL-6 (pg/mL)	1.4±0.8	2.0±0.8	-0.593	-0.922	-0.264	<0.001
SOD (IU/mL)	13.5±1.8	12.6±2	0.942	0.134	1.751	0.006
MDA (µmol/L)	0.9±0.2	1±0.2	-0.121	-0.202	-0.040	0.001
Cystatin-C (mg/L)	0.8±0.1	0.9±0.1	-0.056	-0.103	-0.009	0.016

FSH: Follicle-stimulating hormone, LH: Luteinizing hormone, E2: Estradiol, TSH: Thyroid stimulating hormone, LDL: Low density lipoprotein, HDL: High density lipoprotein, TNF-α: Tumor necrosis factor-alfa, IL-1β: Interleukin-1 beta, IL-6: Interleukin-6, SOD: Superoxide dismutase, MDA: Malondialdehyde, PCOS: Polycystic ovary syndrome, OR: Odds ratio, CI: Confidence interval

Table 3. Univariate and multivariate logistic regression analysis to identify possible risk factors that may affect the PCOS group

	Multivariate					Univariate				
	B	p	OR	95% CI		B	p	OR	95% CI	
Age (years)	-0.060	0.515	0.9	0.8	1.1	-0.099	0.044	0.9	0.8	1.0
TSH (mIU/L)	-0.843	0.070	0.4	0.2	1.1	-0.678	0.009	0.5	0.3	0.8
TNF-α (pg/mL)	0.152	0.004	1.2	1.1	1.3	0.122	0.000	1.1	1.1	1.2
IL-1β (pg/mL)	16.549	0.017	1.1	1.0	1.3	5.765	0.000	0.0	1.1	1.2
IL-6 (pg/mL)	1.354	0.033	3.9	1.1	13.5	1.377	0.018	0.3	1.5	5.9
SOD (IU/mL)	-0.194	0.364	0.8	0.5	1.3	-0.247	0.027	0.8	0.6	1.0
MDA (µmol/L)	1.364	0.528	3.9	0.1	268.8	3.229	0.006	25.2	2.6	247.9
Cystatin-C (mg/L)	11.630	0.012	11.7	2.8	98.1	4.764	0.025	117.2	1.8	7518.4

TSH: Thyroid stimulating hormone, TNF-α: Tumor necrosis factor-alfa, IL-1β: Interleukin-1 beta, IL-6: Interleukin-6, SOD: Superoxide dismutase, MDA: Malondialdehyde, PCOS: Polycystic ovary syndrome, OR: Odds ratio, CI: Confidence interval

of Cystatin-C and inflammatory, oxidant, and antioxidant mediators was evaluated, it was observed that there was a significant correlation with the increase in IL-6 and decrease in SOD.

Studies have shown that Cystatin-C is a good predictor of cardiovascular events[16,17]. It has been reported that it may be an indicator of future cardiovascular risk in women with PCOS[11]. Çınar et al.[18] stated that increased Cystatin-C levels in patients with PCOS are an early indicator of negative clinical outcomes. Statistically significant negative outcomes in the PCOS group in this study were BMI, WHR, FS, triglyceride, LDL, total cholesterol, estradiol, dehydroepiandrosteno-sulphate, free testosterone, LH, high sensitive C-reactive protein. Gozashti et al. [10] also found high Cystatin-C levels in patients with PCOS. In our study, there was no significant difference between the two groups in terms of fasting glucose, fasting insulin and lipid levels, which are cardiovascular risk factors, while Cystatin-C was found to be high regardless of these risk factors.

TNF-α, IL-1β, IL-6 are markers that show inflammation. Studies have shown that TNF-α is higher in women with PCOS

Table 4. Correlation analysis between Cystatin-C and inflammatory and oxidative markers

	Cystatin-C (mg/L)	
	r	p
TNF-α (pg/mL)	0.056	0.590
sIL-1β (pg/mL)	-0.030	0.775
IL-6 (pg/mL)	0.214	0.037
SOD (IU/mL)	-0.280	0.006
MDA (µmol/L)	0.132	0.199

TNF-α: Tumor necrosis factor-alfa, IL-1β: Interleukin-1 beta, IL-6: Interleukin-6, SOD: Superoxide dismutase, MDA: Malondialdehyde

than in the healthy population[19]. TNF-α has been particularly associated with insulin resistance and hyperandrogenemia and is higher in follicular fluid than in serum[20-22]. It has also been stated that high TNF-α levels in patients with PCOS cause the development of type 2 diabetes mellitus (type 2 DM), infertility, atherosclerosis and some cancers[23].

Other cytokines known to increase in PCOS are IL-1β and IL-6. It is thought that *IL-1β* gene activation, which plays a key role in the inflammatory response, may affect steroidogenesis in granulosa cells[24]. It has also been reported that increased IL-1β causes follicular atresia and inhibits oocyte maturation[25]. In the study of Alkhuriji et al.,[26] it was observed that IL-1β levels were high in patients with PCOS with obesity. The increase in IL-1β in these patients is thought to be due to anovulation[27]. It has been shown that IL-6 levels, one of the inflammatory cytokines, are increased especially in patients with PCOS with insulin resistance[28]. IL-6 is thought to have proinflammatory properties that cause insulin resistance[29]. Additionally, it has been observed that insulin resistance and obesity stimulate *TNF-α* and *IL-6* gene expression in adipose tissue in patients with PCOS[30]. Although there was no statistical difference in BMI and WHR between the PCOS and control groups in our study, TNF-α, IL-1β, and IL-6 were found to be statistically significantly higher in the PCOS group. These mediators were independently elevated in patients PCOS when performed in a multivariate analysis. This indicates that inflammation plays an important role in the pathophysiology of PCOS. As it is known, proinflammatory mediators increase the risk of cardiovascular diseases[31].

MDA is an indicator of intracellular and cell membrane damage, and lipid peroxidation[32]. SOD is one of the major antioxidant enzymes that neutralizes free oxygen radicals[33]. They are mediators that show oxidative stress in patients with PCOS[5]. It is stated that insulin resistance, obesity, dyslipidemia, and hyperandrogenism seen in patients with PCOS increase MDA levels and decrease SOD levels[34]. Increased MDA levels are an indicator of lipid oxidation and this is a risk factor for cardiovascular diseases[5]. Studies on SOD levels are conflicting. While studies have shown that it decreases in patients with PCOS, there are also studies indicating that SOD levels increase in response to increased oxidant levels in the circulation[5,35]. Polat and Şimşek[36] reported in their study that Turkish women with PCOS had mutations in the *SOD-1* and *SOD-2* genes and did not have sufficient antioxidant capacity. In our study, MDA was found to be high and SOD to be low in patients with PCOS.

In our study, there was no difference between the two groups in terms of BMI, WHR, fasting glucose, fasting insulin, and lipid levels, while a significant difference was found between Cystatin-C, inflammatory, oxidant, and antioxidant markers. This shows that inflammation and oxidant-antioxidant pathway are affected independently by obesity, metabolic syndrome, and insulin resistance. Additionally, although routine cardiovascular risk factors seem to be normal, high Cystatin-C levels made us think that these mediators may be related. In the correlation analysis performed for this purpose, the increase in Cystatin-C was correlated with the increase in IL-6 and the decrease in the SOD level. Gozashti et al.[10], in their study, no relationship was found between elevated Cystatin-C and inflammatory mechanisms in patients with PCOS. There is no other study in the literature examining this relationship. Clarification of this relationship is also important in terms of treatment. Polat and Şimşek[36] who detected *SOD-1* and *SOD-2* gene mutations in patients with PCOS, suggested adding antioxidant supplementation to the treatment due to decreased antioxidant capacity. When the relationship between IL-6 and SOD and Cystatin-C is evaluated, it may be necessary to add antioxidant supplements and anti-inflammatory agents to the treatment for cardiovascular protection. However, more studies are needed to include them in routine treatment.

Study Limitations

Our study has some limitations. A limitation is that the patient sample is too small and the PCOS cannot be divided into subgroups. Not looking for oxidant-antioxidant markers other than MDA and SOD may be another limitation. In addition, we did not apply antioxidant supplements and anti-inflammatory treatments to these patients. Therefore, we do not have post-treatment results. However, even if BMI, WHR, fasting glucose, fasting insulin, and lipid values are not different from the control group, it is important to show the elevation of Cystatin-C in these patients and to correlate this elevation with IL-6 and SOD.

Conclusion

Our study showed that Cystatin-C levels were high in patients with PCOS, even though there was no difference between the control group and the PCOS groups in terms of other cardiovascular risk factors. It is also the only study showing the relationship between increased Cystatin-C levels and IL-6 and SOD. This result may be effective in the treatment plan of the patients. However, our results should be confirmed with studies conducted with more patients.

Ethics

Ethics Committee Approval: The Yozgat Bozok University Local Ethical Committee approved the present study (2017-KAEK-189_2021.12.29_02).
Informed Consent: Informed consent was obtained from all participants.
Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: A.Y.G., Concept: Ö.Ö.B., A.Y.G., Design: Ö.Ö.B., Data Collection or Processing: Ö.Ö.B., Analysis or Interpretation: A.Y.G., D.A.K., Literature Search: Ö.Ö.B., A.Y.G., D.A.K., Writing: Ö.Ö.B., D.A.K.
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The protective effect of cilostazol on experimental ischemia/reperfusion injury in rats ovaries on in vitro fertilization outcomes

Silostazolün sıçan yumurtalığında deneysel iskemi/reperfüzyon hasarına karşı in vitro fertilizasyon sonuçlarına koruyucu etkisi

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Abstract

Objective: Ovarian torsion decreases ovarian reserve because of ischemic and reperfusion damage it causes. In this study, we investigated the protective effect of cilostazol (CIL) on experimental ischemia (I) and ischemic-reperfusion (I/R) damage in rat ovaries with in vitro fertilization (IVF) results.

Materials and Methods: Forty-eight adult female Sprague-Dawley albino rats were randomly assigned to 6 groups with 8 animals in each group: Sham (S), I, I/R, S + CIL, I + CIL and I/R + CIL. The I groups were subjected to bilateral adnexal torsion for 3 h, while the I/R and I/R + CIL groups received subsequent detorsion for 3 h. Twenty-two mg/kg of CIL was given via oral gavage 30 min before surgery on the I (I+ CIL) or reperfusion (I/R + CIL) groups. Oocytes were collected before the IVF procedure and after ovulation induction with 150-300 IU/kg pregnant mare serum gonadotropin.

Results: The metaphase oocytes reached their highest value of 4.73±0.96 in the S+ CIL group and reached their lowest value of 0.51±0.55 in the I/R group. There were statistically significant differences in the number of second-day embryos among the I, I+ CIL, and I/R and I/R+ CIL groups (p=0.000). When the groups were compared in terms of Anti-Müllerian hormone change, the highest decrease was observed in the I and I/R groups.

Conclusion: CIL pretreatment before surgery has a protective effect against I and I/R in rats with ovarian torsion.

Keywords: Reperfusion injuries, ovarian torsion, in vitro fertilization, cilostazol

Öz

Amaç: Over torsiyonu, neden olduğu iskemik ve reperfüzyon hasarı sonucunda over rezervinde azalmaya neden olur. Bu çalışmada, rat overlerinde oluşturulan deneysel iskemi (I) ve iskemik-reperfüzyon (I/R) hasarına karşı silostazolün (CIL) koruyucu etkisinin in vitro fertilizasyon (IVF) sonuçları ile araştırılması amaçlanmıştır.

Gereç ve Yöntemler: Kırk sekiz yetişkin dişi Sprague-Dawley albino ratı, her grupta 8 hayvan bulunan 6 gruba rastgele atandı: Sham (S), I, I/R, S + CIL, I + CIL ve I/R + CIL. I grupları 3 saat boyunca bilateral adneksiyal torsiyona maruz kalırken, I/R ve I/R + CIL grupları 3 saat boyunca detorsiyon alındı. I (I + CIL) veya reperfüzyon (I / R + CIL) gruplarında ameliyattan 30 dakika önce oral gavage yoluyla 22 mg/kg CIL verildi. IVF işlemi öncesi ile 150-300 IU/kg gebe kısrak serum gonadotropin ile ovülasyon indüksiyonu yapıldıktan sonra oositler toplandı.

PRECIS: The use of cilastazole has a protective effect against ovarian torsion, which is one of the important causes of infertility by causing a decrease in ovarian reserve.

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Bulgular: Metafaz oositleri S+ CIL grubunda 4,73±0,96 ile en düşük değerlerine ulaşırken I/R grubunda 0,51±0,55 ile en düşük değerlerine ulaştılar. I, I+ CIL ve I/R ve I/R+ CIL grupları arasında ikinci gün embriyo sayısında istatistiksel olarak anlamlı farklılıklar vardı (p=0,000). Gruplar kendi içlerinde Anti-Müllerian hormon değişimi açısından karşılaştırıldığında, en yüksek düşüşün I ve I/R gruplarında olduğu gözlenmiştir.

Sonuç: Over torsiyonunda, ratlarda cerrahi uygulama öncesi CIL ön tedavisinin I ve I/R'ye karşı koruyucu etkisi vardır.

Anahtar Kelimeler: Reperfüzyon hasarları, over torsiyonları, in vitro fertilizasyon, silostazol

Introduction

Adnexal torsion is defined as twisting the ovary on its own or with the fallopian tube in the axis between the infundibulopelvic ligament and the utero-ovarian ligament[1]. Ovarian torsion, which is an important gynecological emergency, is most common in the reproductive years, with annual prevalence of approximately 2-6%[2]. Although there are many risk factors for this condition, the most common ones are ovulation induction, ovarian cysts, endometriosis, and hyperlaxity of the ovarian ligaments[3]. If not diagnosed and treated promptly, ovarian torsion causes ovarian necrosis and irreversible damage to the tissue. The duration of torsion is significant for tissue damage (loss of ovarian function) and the resulting decrease in infertility. While I injury is a possible cause of tissue damage because of torsion, post-detorsion ischemic-reperfusion (I/R) injury also contributes to tissue damage due to overproduction of reactive oxygen species (ROS)[4].

Cilostazol (CIL) is a phosphodiesterase III inhibitor that increases cyclic adenosine monophosphate levels, leading to protective effects, antioxidant activity and anti-apoptosis effects in endothelial cells[5]. It has been shown that CIL reduces the damage caused by I and R in various tissues such as the myocardium and kidney[6,7].

When the literature is evaluated, it is seen that there are studies with histopathological or biochemical markers evaluating the protective effects of many agents in preventing I- and I/R-related damage in the ovarian tissue because of torsion[8-10]. It is seen that these studies give an idea about the future results of adnexal torsion on variables such as ovarian morphology and follicular numbers, and they do not provide sufficient information about the expected follicular development in the future, the number of oocytes that can be obtained and the embryo formed after fertilization.

Therefore, in this study, which is supported by the comparison of serum Anti-Müllerian hormone (AMH) levels, which is a reliable ovarian reserve test method, it was planned to obtain oocytes after ovulation induction in rats, to evaluate fertilization and embryo quality by in vitro fertilization (IVF).

In this experimental study, it was aimed to evaluate the protective effect of CIL on the formation of I and I/R damage in the ovaries by using IVF results and serum AMH levels.

Materials and Methods

Ethics and Animals

The study was conducted out in the Sakarya University SUDETAM laboratory with the authorization and approval of the Experimental Animals Ethics Committee of Sakarya University, based on the European Commission Directive 86/609/ECC guide protocol, with the decision dated 05/05/2021 and numbered 27.

The study comprised 48 virgin Sprague-Dawley albino rats (weighing from 220 to 260 g). For one week before the study, the animals were maintained under appropriate humidity (50±5%) and heat regulation (22±2 °C) over a 12-hour light/dark cycle.

Surgical Procedures and Experimental Protocol

The rats were randomly divided into six groups of 8 animals each: S operation, I (3 h), I/R (3 h ischemia plus 3 h reperfusion), S, S + CIL, I + CIL and I/R + CIL (3 h ischemia and 3 h reperfusion). Rats in the CIL groups (S + CIL, I + C, I/R + CIL) were given 12 mg/kg of cilostazol via oral gavage 30 min before surgery. A blood sample (AMH1) was drawn from each rat to measure the serum AMH level before operations began.

Before surgery, rats were anesthetized with ketamine hydrochloride (60 mg/kg of Ketalar; Eczacibasi, Istanbul, Turkey) and xylazine hydrochloride (7 mg/kg of Rompun; Bayer Türk Ilaç Ltd., Istanbul, Turkey) under sterile conditions. During surgery, the rats were covered with a sterile drape in the dorsal recumbent position. Uterine horns and adnexa were observed after entering the abdomen by making a longitudinal 2 cm long midline incision in the lower abdomen of the rats. In the S group, the abdominal folds were closed again with 3/0 silk sutures after 2 min of observation. In the ischemia group, the ovarian pedicles on both sides were rotated 360 ° clockwise and fixed to the abdominal wall with 5/0 silk sutures, and the abdominal folds were closed with 3/0 silk sutures. In the I/R group, after the above-mentioned 3-hour ischemia period, after the abdominal layers were reopened, bilateral adnexal detorsion was performed by removing the torsion sutures. In the S + CIL group, the S operation was performed as described. In the I + CIL group, adnexal torsion was performed. In the I/R + CIL group, sequential bilateral adnexal torsion and detorsion were performed.

Ovulation induction was performed in rats that had undergone at least three consecutive estrous cycles as determined by daily vaginal smear. All rats with ovulation induction were sacrificed to collect oocytes. To compare meiotic progression in classifying oocytes according to germinal vesicle (GV), metaphase I (MI), and metaphase (MII) stages, the mean time for each stage of nuclear progression Sirard et al.[11] the previously described method was used.

HTF (Human tubal fluid) medium (Cat. no. 90166, Irvine Scientific, USA) was used for sperm preincubation, fertilization

and embryo transfer. Embryos were washed by passing through a 35-mm culture dish (Nunc, Cat. No.63754, Denmark) covered with liquid paraffin oil (Cat. No. 9305, Irvine Scientific, USA), and maintained at 37 °C under 5% CO_2 in humidified air overnight.

Following administration of 150 to 300 µIU/kg [Chronogest/ pregnant mare serum gonadotropin (PMSG), Intervet, Istanbul, Turkey] using an intraperitoneal (i.p.) injection as an ovulation stimulation protocol, 150-300 µIU/kg human chorionic gonadotropin after approximately 48 h; (Gonatropin, Chorulon® Intervet, Istanbul, Turkey) was applied. PMSG was administered at a dose of 15 IU 17 to 19 h after administration[12]. Before anesthesia, all rats were weighed, and intramuscular administered of 50 µmg/kg ketamine hydrochloride (Ketalar; Eczacıbaşı Warner Lambert İlaç Sanayi, Levent, Istanbul, Turkey) and 7 µmg/kg xylazine hydrochloride (Rompun, Bayer Şişli, Istanbul, Turkey) were used for the procedure.

After the anesthetized rats were placed in suitable conditions, second blood samples were collected to measure the serum AMH2 level. After the ovaries were properly dissected and removed, oocytes were collected. Oocytes were cultured for one day before being placed in an incubator in HTF medium supplemented with human serum albumin 4 mg/mL, 37 °C, and 5% CO_2. After transferring oocytes and capacitive sperm (approximately 1x106 mL-1) to fertilization droplets, fertilization was controlled and the resulting embryos were followed up to the two-cell stage[12].

A male rat was sacrificed with appropriate anesthesia ketamine hydrochloride (60 mg/kg Ketalar; Eczacıbaşı, Istanbul, Turkey) and xylazine hydrochloride (7 mg/kg Rompun; Bayer Türk Ilaç Ltd., Istanbul, Turkey) just before oocyte retrieval. A vertical incision was made in the abdomen of this rat and the abdomen was entered and the male reproductive system was observed. The bilateral epididymis was separated from the testicles with appropriate dissection and transferred to HTF medium. The obtained epididymis was stripped by appropriate dissection and the obtained sperm was transferred to Petri dishes and incubated at 37 °C for 30 min before IVF procedure[13].

The collected oocytes were cultured as described above after washing three times with HTF medium before insemination. Seven to eight hours after insemination of oocytes, evaluation for sperm penetration or pronuclear formation was performed under an inverted microscope to identify polyspermic fertilization or parthenogenetic embryos (approximately 6.5% of total). Following this process, the culture was continued for another 20 h and the embryos at the 2 cell stage were counted[14].

Enzyme-linked immunosorbent assay (ELISA) was used in the evaluation of serum concentrations of AMH in accordance with the application guidelines of the manufacturer (BT LAB Biotech Co. Ltd., Shanghai Cat. No: E0456Ra). The sensitivity of the kit used in the study for the AMH value was reported as 0.1 ng/mL to 40 ng/mL by the company.

Statistical Analysis

The Kolmogorov-Smirnov test was used for the normal distribution of the data, while the Kruskal-Wallis test was used to compare more than two variables that did not show normal distribution. Mann-Whitney U test was used for pairwise comparisons between groups for differing parameters. Since AMH values showed a normal distribution, dependent groups were compared using the paired sample test. After all, results were evaluated as mean ± standard deviation, the results with a $p<0.05$ value were considered statistically significant.

Results

Total Oocyte Count

The number of oocytes collected from the rats showed statistically significant differences between the groups (p=0.000). The group with the highest oocyte collection, with an average of 8.75±1.35, was the S group, while the lowest number of oocytes was seen in the I/R group with 2.37±1.32. When the groups were compared after cilostazol application, the number of oocytes collected did not differ between the S and S+CIL groups (p>0.05).

However, it was observed that the number of oocytes collected in the I and I+CIL (p=0.021) and I/R and I/R+CIL (p=0.003) groups increased with the effect of cilostazol (Figure 1), so cilostazol increased the number of oocytes collected.

MII, MI and GV Oocyte Counts

Statistically significant differences in MII, MI and GV oocytes were also seen between the groups (p=0.000 for all three parameters). It was observed that cilostazol administration did not create a statistically significant difference between the S and S+CIL groups for MII oocyte counts (p>0.05). It was observed that cilostazol administration had a positive effect on the increase of MII oocyte numbers in groups I and I/R. Statistically significant differences were observed between I and I+CIL (p=0.001) and I/R and I/R+CIL (p=0.000) groups. It was also observed that CIL application did not create a difference between the study groups in terms of M1 oocyte numbers. It was seen that the number of GVs collected decreased significantly after CIL application. There was a statistically significant difference between the S and S+CIL (p=0.011), the I and I+CIL (p=0.003), and the I/R and I/R+CIL (p=0.036) groups (Figure 1).

Second Day Embryo Counts

When the groups were compared in terms of the number of embryos obtained on the second day, statistically significant differences were found (p=0.000). While there was no statistically significant difference in the number of embryos obtained on the second day between the S and S+CIL groups (p>0.05), it was observed that the number of embryos on the second day was higher in the I+CIL and I/R+CIL groups compared to the groups without CIL. When the groups were compared among

themselves, there was a statistically significant difference in p values between I and I+CIL groups, with p=0.001, p=0.000 between I/R and I/R+CIL groups (Figure 1).

AMH Concentrations

There was no statistically significant difference in AMH1 concentrations between the study groups (p>0.05). To compare the effects of CIL application on AMH, the correlation between AMH values of S+CIL, I+CIL and I/R+CIL groups were examined. For this, the paired-samples t-test was applied for the correlation of binary groups. It was observed that CIL application had no effect on the AMH1 and AMH2 values of the S+CIL group (p>0.05), while it was observed that CIL application had positive effects on the AMH concentration in the I+CIL and I/R+CIL groups. A high degree of correlation was observed when comparing AMH values in the I+CIL and I/R+CIL groups (p=0.000 for both groups) (Figure 2).

Discussion

In this study, the aim was to evaluate the efficacy of cilostazol and detorsion in preserving ovarian reserves and structure. Pretreatment with CIL is effective in preserving ovarian reserves after post-torsion injury. This is the first study to evaluate IVF outcomes in predicting ovarian reserve in rat ovaries and provide evidence of the protective effect of CIL on I and I/R injury. To apply a detorsion procedure to preserve ovarian reserves in cases of torsion. Misdiagnosis or delay in treatment in these patients affects fertility in the long term by causing serious losses in the ovarian reserve.

Various physiopathological mechanisms explain the causes of tissue damage due to ovarian torsion and detorsion[15,16]. I/R injury usually occurs due of increased ROS production due to activated complement proteins and other inflammatory components around the inflammation site[17]. It was observed

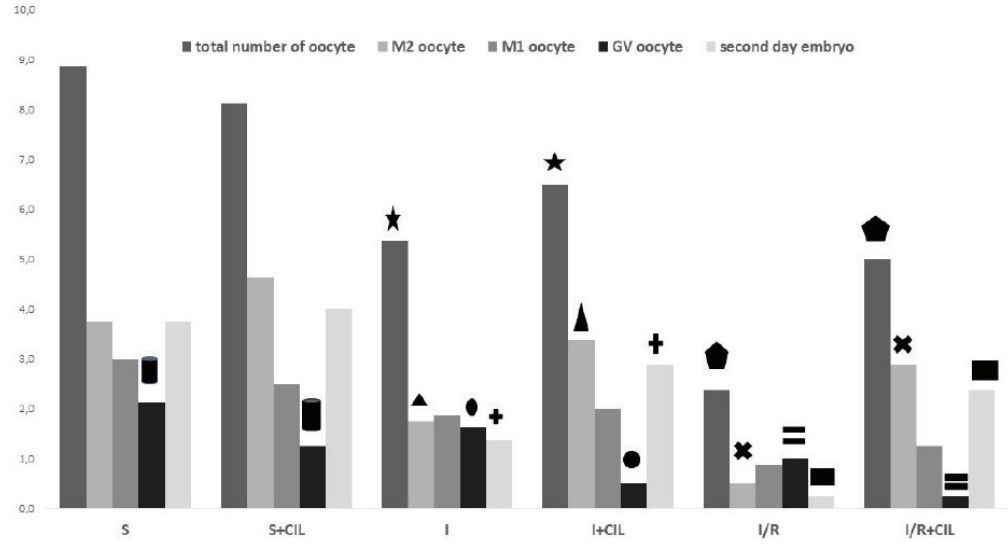

Figure 1. A comparison of the study groups' total oocyte count, germinal vesicle (GV), metaphase I (MI), metaphase II (MII) oocyte counts, and second-day embryo counts. Statistical analysis between all groups was performed with a Kruskal-Wallis test. Pairwise comparisons were made with the Mann-Whitney test. The black cylinder indicates the statistically significant difference between the sham (S) and S+ (cilostazol) CIL groups. There was a statistically significant difference between the black star, triangle, circle and plus sign ischemia (I) and I+CIL groups. There was a statistically significant difference between the black pentegon, cross, equalitiy and rectangle ischemia reperfusion (I/R) and I/R+CIL groups

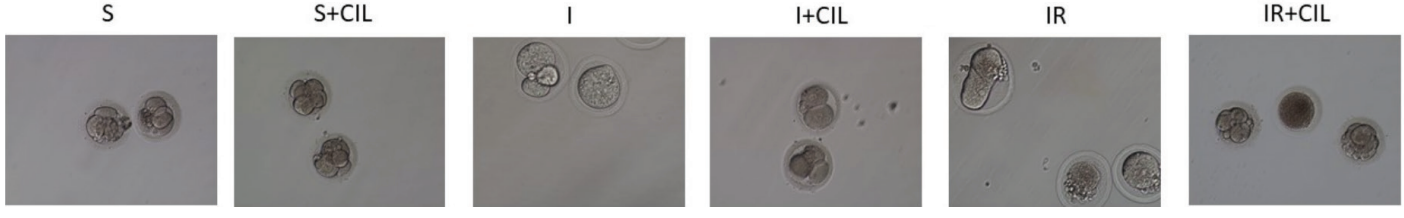

Figure 2. Comparison of second day embryos between groups. Second-day embryos of the groups are seen at 100x magnification. The second-day embryo counts and embryo quality in the sham (S) and S+ cilostazol (CIL) groups were quite good compared to the other groups. It is seen that the quality and number of embryos on the second day are significantly better in the Ischemia (I) + CIL and ischemia reperfusion (I/R) + CIL groups compared to the I and I/R groups, respectively

that ovarian injury was more severe in the detorsion group than in the torsion group, which followed reports in published literature[8,18]. For this reason, it is important not only to prevent I in cases of ovarian torsion but also to prevent I/R damage that will occur because of detorsion applied in the treatment.

CIL, a phosphodiesterase inhibitor that suppresses platelet aggregation and has a vasodilator effect, plays an important role in modulating the oxidant-antioxidant system to reduce I/R damage[19]. Because of these properties, CIL are widely used in treating chronic peripheral artery disease and for treating ischemic coronary artery disease[20]. CIL pretreatment significantly reduces IL-6 levels in long-term use, reducing lipid peroxidation and ROS in I/R injuries[21].

CIL reduces drug-induced nephrotoxicity with antioxidant and anti-apoptotic activities[19]. CIL effectively repairs tissue damage caused by I/R and reduces oxidant stress in heart tissue[22]. Experimental studies have demonstrated that CIL produced a protective effect against I injury in animal models when used for skeletal muscle[6,23].

In published literature, it has been shown with histopathological and serum biochemical markers that many treatment agents have a protective effect against I and I/R in cases of ovarian torsion[8,24,25]. In terms of ovarian reserve, it is seen that studies are evaluating the number of different types of follicles that affect ovarian reserves[26]. These results cannot give a clear idea about the number of oocytes that can be obtained in the later reproductive period and the number and quality of embryos formed after fertilization of these oocytes. Additionally, these results do not fully reflect the long-term effectiveness of the protective effect of these treatment agents in terms of reproductive health.

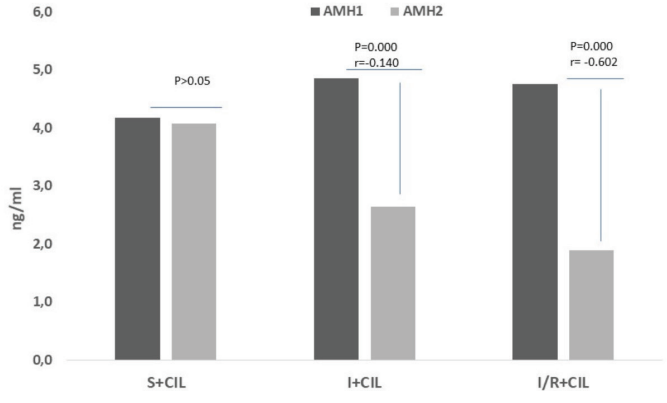

Figure 3. Comparison of Anti-Müllerian hormone (AMH) levels after cilostazol administration between groups via an AMH1 and AMH2 correlation plot between the sham (S)+ cilostazol (CIL), Ischemia (I) +CIL and Ischemia reperfusion (I/R) +CIL groups (mean and 95% confidence interval). There was no correlation between AMH1 and AMH2 values in the S+CIL group (P>0.05). A high level of correlation was observed between AMH1 and AMH2 values in the I+SI and I/R+SI groups. Analysis was done with a paired sample test. P<0.05 was considered statistically significant

In this study, while the results obtained with ovulation induction in the cycle after the treatment process show the long-term effects of the protective effect on the follicles constituting the ovarian reserve more significantly, the evaluation of the fertilization of the obtained oocytes and the comparison of the obtained two-cell embryo numbers will be important in estimating the clinical results.

In this study, the total number of oocytes obtained in the I and I/R groups that underwent CIL, and especially the MII oocyte counts, were higher than those in the I and I/R groups (Figure 1). While these results showed the protective effect of CIL on the follicles, similar results were obtained in the number of two-cell embryos, and the high number of embryos after fertilization suggests a protective effect that also affects the oocyte quality (Figure 1, 2). When the groups were evaluated in terms of total oocyte counts, the protective effect of CIL was seen in the I+CIL and I/R+CIL groups compared with the I and I/R groups. Since the results of embryos obtained by ovulation induction and subsequent IVF were evaluated in this study, the main limitation of the study is that the natural cycle results are not known.

To support IVF results, AMH levels, a current and reliable marker of ovarian reserve, were measured and compared in the preoperative and postoperative periods in our study. Supporting the difference in oocyte numbers, the decrease in AMH values was significantly higher in the I and I/R groups compared with the I+CIL and I/R+CIL groups (Figure 3).

Study Limitations

Other limitations of the study are that it is not a human study and there are no results about embryo implantation, the pregnancy rate obtained and live birth after pregnancy, which are other parameters that provide information about reproductive status. We think that this study is the first experimental rat study in which the effects of CIL on ovarian torsion and detorsion-related damage were investigated by evaluating IVF results, and it showed that it was effective in reducing ischemic and detorsion-induced ovarian damage.

Conclusion

In a rat ovarian torsion model of ovarian ischemia, cilostazol pretreatment was associated with viable oocytes and successful embryo implantation. Further studies including human subjects will are required to confirm these findings.

Ethics

Ethics Committee Approval: The study was conducted out in the Sakarya University SUDETAM laboratory with the authorization and approval of the Experimental Animals Ethics Committee of Sakarya University, based on the European Commission Directive 86/609/ECC guide protocol, with the decision dated 05/05/2021 and numbered 27.

Informed Consent: Not necessary.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: Ö.B., O.K., Ö.D., M.S.B., H.Ç., E.Ç., Design: H.Ç., Ö.B., M.S.B., O.K., Ö.D., E.Ç., Data Collection or Processing: Ö.B., M.S.B., O.K., H.Ç., Ö.D., E.Ç., Analysis or Interpretation: E.Ç., Ö.B., M.S.B., O.K., H.Ç., Ö.D., Literature Search: M.S.B., Ö.B., O.K., H.Ç., Ö.D., E.Ç., Writing: M.S.B., Ö.B., O.K., H.Ç., Ö.D., E.Ç.

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

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Quantitative serum determination of CD3, CD4, CD8, CD16, and CD56 in women with primary infertility: The role of cell-mediated immunity

Primer infertilitesi olan kadınlarda CD3, CD4, CD8, CD16 ve CD56'nın kantitatif serum tayini: Hücre aracılı bağışıklığın rolü

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Abstract

Objective: Cellular adaptive immunity plays an essential role in the etiology of primary infertility. This study aimed to measure the T-lymphocyte subpopulations and natural killer (NK) cells in infertile women compared with healthy ones.

Materials and Methods: From January to September 2021, we conducted this cross-sectional study among women with primary infertility, and healthy women were referred to Isfahan Fertility and Infertility Center affiliated with Najafabad University of medical sciences in Isfahan, Iran for immunological investigations. For each person, we determined quantitative serum measurements of CD3, CD4, CD8, CD4/CD8, CD16, CD56, and CD56+16.

Results: This study included one hundred and fifty-one infertile women with a mean age of 31.4±4.7 years and 46 healthy women with a mean age of 31.5±3.4 years. Compared to the controls, immunophenotyping findings in infertile patients revealed a significant drop in CD8 T cells [p=0.01, 95% confidence interval (CI) 0.53 to 4.57] and the percentage of CD 56 NK cells (p=0.005, 95% CI 0.74 to 4.03) in infertile patients.

Conclusion: Despite having a normal quantity of CD3 T cells, infertile women had lower CD8 T cells and CD56 NK cells than the controls. More studies are needed to confirm the role of cell-mediated assessments as a screening test in patients with primary infertility.

Keywords: Infertility, cellular immunity, flow cytometry, female infertility, NK cells, T cells

Öz

Amaç: Hücresel adaptif immünite, primer infertilite etiyolojisinde önemli bir rol oynar. Bu çalışma, infertil kadınlarda ve sağlıklı kadınlarda T-lenfosit alt popülasyonlarını ve doğal öldürücü (NK) hücrelerini ölçmeyi ve bunların iki grup arasında kıyaslanmasını amaçlamaktadır.

Gereç ve Yöntemler: Bu kesitsel çalışmayı Ocak-Eylül 2021 tarihleri arasında İran'ın İsfahan kentindeki İsfahan Tıp Bilimleri Üniversitesi'ne bağlı İsfahan Doğurganlık ve Kısırlık Merkezi'ne immünolojik incelemeler için sevk edilen primer infertilitesi olan kadınlar ve sağlıklı kadınlar üzerinde gerçekleştirdik. Her kişide CD3, CD4, CD8, CD4/CD8, CD16, CD56 ve CD56+16'nın kantitatif serum ölçümlerini yaptık.

Bulgular: Bu çalışmaya yaş ortalaması 31,4±4,7 yıl olan 115 infertil kadın ve yaş ortalaması 31,5±3,4 yıl olan 46 sağlıklı kadın dahil edildi. Kontrollerle karşılaştırıldığında, infertil hastalarda immünofenotipleme bulguları, CD8 T hücrelerinin sayısında [p=0,01, %95 güven aralığı (GA) 0,53 ila 4,57] ve CD 56 NK hücrelerinin yüzdesinde (p=0,005, %95 GA 0,74 ila 4,03) önemli bir düşüş ile uyumluydu.

Sonuç: Normal miktarda CD3 T-hücresine sahip olmalarına rağmen, infertil kadınlarda kontrollere göre daha düşük CD8 T hücreleri ve CD56 NK hücreleri vardı. Primer infertilitesi olan hastalarda bir tarama testi olarak hücre aracılı değerlendirmelerin rolünü doğrulamak için daha fazla çalışmaya ihtiyaç vardır.

Anahtar Kelimeler: Kısırlık, hücresel bağışıklık, flow sitometri, kadın infertilitesi, NK hücreleri, T hücreleri

PRECIS: The rate of peripheral CD8 T cells and CD56 NK cells significantly decreased in women with infertility compared to the controls in our study.

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Introduction

Primary infertility (PI) is defined as a woman's inability to become pregnant after one year of regular intercourse without using contraception methods, as well as normal results for semen analysis, ovulation tests, and tubal patency. Based on a World Health Organization report, PI affects around one-fourth of all couples worldwide. According to a new theory, PI can be caused by immunological disorders, including a deficiency in the humoral or cellular immune systems[1-4].

Cell-mediated immunity is an immune response that does not involve antibodies but involves the production of T-lymphocytes and the activation of macrophages and natural killer (NK) cells. It secretes various cytokines in response to an antigen or immunogen[5,6].

CD3 is a characteristic marker for recognizing T cells, T-cell receptors, and a ubiquitous T-cell receptor complex member. CD3 T cells can be divided into two predominant types by the expression of surface molecules of CD4 ($CD4^+$ or T-helper cells) and CD8 ($CD8^+$ or cytotoxic T cells). $CD4^+$ or T-helper cells (TH) may be differentiated into two main categories: TH_1 cells, which produce interferon-gamma and lymphotoxin-alpha, and TH_2 cells, which produce IL-4, IL-5, and IL-13. As a third group, T helper 17 cells (TH17), which secrete IL-17, were found. $CD8^+$ or cytotoxic T-cells generally produce interferon-gamma; they may also be differentiated into two main categories: TC_1 cells, TC_2 cells, and newly discovered TC_{17} that secretes Interleukin 17[6,7].

NK cells are granular lymphocytes with NK- specific CD antigens, CD16 and CD56, and a potent source of interferon-gamma. Peripheral NK cells and uterine NK cells are two types of NK cells. Peripheral NK cells circulate in the bloodstream, but uterine NK cells lack the same destructive ability as peripheral NK cells[8,9].

Infertility is a serious problem that can ruin a couple's life. Although the interest in considering the disturbance of immunologic factors for the occurrence of PI has recently increased, there are limited reports of cell-mediated immunity in the serum of infertile females. This study measured the serum levels of CD3, CD4, CD8, CD16, and CD56 in infertile females compared with the control group in Isfahan, Iran.

Materials and Methods

From January to September 2021, this cross-sectional study involved voluntary females with PI aged 18 to 42 who were referred to Isfahan Fertility and Infertility Center, affiliated with Najafabad University of medical sciences in Isfahan, Iran, for immunological investigations. PI diagnosis was based on the lack of abnormality in the semen analysis and the normal assessment of ovulation and fallopian tubes in the women. After the approval of the study protocol by the Ethics Committee of Najafabad University of Medical Sciences (IR.IAU.NAJAFABAD.REC.1399.119), written informed consent was obtained from the patients.

The women were excluded if they had at least one successful pregnancy, uncontrolled diabetes, uncontrolled hypertension, autoimmune diseases, malignancy, or an immune deficiency disorder. Women who had previously had an abortion were included if it had been at least a year since the abortion. Forty-eight unrelated healthy women with a history of having at least two children aged from 18 to 42 years were selected as the control group by the non-probability Quota sampling method from the same geographic region.

A questionnaire was designed to collect information about age, duration of the marriage, and the number of abortions. Peripheral blood samples were collected aseptically by venipuncture into ethylenediaminetetraacetic acid collection tubes from all individuals in the case and control groups for quantitative serum determination of CD3, CD4, CD8, CD4/CD8, and CD16, CD56, CD56+16. According to the manufacturer's instructions, a minimum of 5 mL of whole blood is required for the immunophenotypic analysis. Analysis was performed using a FACS Canto II flow cytometer and FACS Diva software (BD Biosciences). Each lymphocyte subpopulation count was expressed in absolute value and as a percentage of total lymphocytes. An example of gating for standard proliferation analysis of lymphocytes in a woman with PI using flow cytometry is shown in Figure 1.

Statistical Analysis

The data were analyzed using SPSS Statistics (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.); correlations were significant if the p-value was less than 0.05. Continuous variables were presented as the mean ± standard deviation. Chi-square, independent sample t-test, and Fisher's exact test were used to determine the mean and compare the mean of CD markers in patients with PI and the control group. The association between independent variables was also assessed 95% confidence interval (CI).

Results

One hundred and fifty-one infertile women with a mean age of 31.44±4.72 years, the age range of 20 to 44 years, and 46 healthy women with a mean age of 31.50±3.39 years, the age range of 22 to 39 years were included in this study. There were no significant differences in age between the two groups (p-value=0.8). All patients were from Isfahan province, central Iran.

The mean period of infertility was 4.7±3.5 years (range: 2 to 16 years) in infertile females. Among enrolled women, 61 (40.4%) had never experienced pregnancy, while 90 (59.6%) had at least one unsuccessful pregnancy. Table 1 represents the mean percentile of T-cell-mediated markers in infertile women compared with control healthy women; the level of CD8 between these two groups was significantly different. The comparison of the mean percentile of natural killer cell markers in infertile women with the control group is shown in Table 2.

Discussion

There are a few studies on the role of T-cell and NK cell marker changes in women with PI. In this study, the immunologic profile showed a significant difference in the percentage of CD 8-T cells and CD 56 NK cells of females with PI without a successful pregnancy and those who had. Compared to the control group, there were no differences in the percentages of CD4 T cells and CD 16 NK cells in patients with infertility.

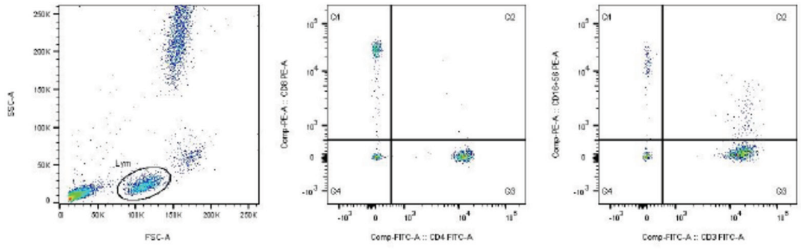
Russell et al.[10] reported focal perivascular aggregation of CD8 T cells in the endometrium of most women with recurrent reproductive failure. Bczkowski and Kurzawa[11] showed that CD4 and CD8-positive cells did not significantly differ in patients treated with intracytoplasmic sperm injection compared with control fertile patients. Lachapelle et al.[12] found that the percentage of endometrial CD8 T-lymphocytes was significantly decreased in cases with recurrent miscarriage. The analysis of the presence of CD8 T cells seems to be a somewhat controversial issue, as our study showed decreased CD8 T cells in patients with PI. Different studies used local endometrial tissue samples to analyze CD8 T cells, whereas our research used peripheral blood. According to Chernyshov et al.[13], no changes in CD8 T lymphocytes were found between endometrial and peripheral blood. All the discrepancies cannot be attributed to the lack of a standardized CD4 measurement location.

The level of CD56 NK cells in women with PI was considerably lower than in controls, confirming that NK cells play a key role in human reproductive performance. According to a recent meta-

Table 1. Comparison of mean percentile of T- cell mediated markers in infertile women with control healthy women

Cell markers	Group	n	Mean	Standard deviation	Minimum	Maximum	95% Confidence Interval		p-value
							Lower	Upper	
CD3, %	case	151	66.14	7.13	34	89	-.1.172	4.587	0.23
	control	46	67.84	7.13	48	78			
CD4, %	case	151	42.01	7.87	22.5	61	-0.464	4.67	0.09
	control	46	44.14	6.84	32	56.5			
CD8, %	case	151	24.01	6.33	3	38	0.533	4.568	0.01*
	control	46	26.56	5.11	17	38.4			
CD4 to 8 ratio	case	151	1.89	0.86	0.8	6.9	-0.447	0.079	0.16
	control	46	1.71	0.50	0.9	3.2			

*: Statistical significant



Immunophenotyping by flow cytometry

CD Marker	Description	Gate	Results		Reference value	
			Relative count (%)	Absolute count (cell/mm³)	Relative count (%)	Absolute count (cell/mm³)
CD3	Pan T cell	Lym	79.4	2717	58-86	550-2202
CD4	T helper cell	Lym	54.67	1871	32-64	365-1437
CD8	T cytotoxic cell	Lym	30	1027	13-40	145-846
CD4:CD8	Th/Tc Ratio		1.8		≥0.9	
CD3-/CD56+	NK cells	Lym	4.76	163	3.5-23	57-611
CD3+/CD56+	NKT cells	Lym	3.47		0.9-15	
CD3-/CD16+	NK cells	Lym	3			
CD3-/CD16+CD56	Total NK cells	Lym	7	239	4-25	57-611
CD3+/CD16+CD56	NKT cells	Lym	10.2		1-15	

Figure 1. Example of gating for standard prolifration analysis of lymphocytes in a woman with primary infertility using flow cytometry

Table 2. Comparison of mean percentile of natural killer cell markers in infertile women with control healthy women

Antibodies	Group	n	Mean	Standard deviation	Minimum	Maximum	95% Confidence Interval		p-value
							Lower	Upper	
CD16, %	Case	151	10.18	5.30	3	31	-0.959	2.394	0.40
	Control	46	10.9	4.03	3	27.6			
CD56, %	Case	151	8.19	5.40	1	31	0.735	4.032	0.005*
	Control	46	10.34	4.00	3	27			
CD16+56, %	Case	151	10.28	6.38	0.8	33	-1.852	2.141	0.82
	Control	46	10.43	4.46	2	28			

*: Statistical significant

analysis of studies that looked at peripheral NK cell levels, the percentage of NK cells in the blood is much higher in women who have recurrent abortions than in those who don't[14]. However, a systematic review by Tang et al.[15] reported that the prognostic value of measuring peripheral NK cell parameters remains unclear, and more studies are needed to confirm the role of NK cell measurement as a predictive test for screening in infertility. Chernyshov et al.[13] revealed that the level of NK cells was higher in the endometrium samples compared to peripheral blood. It's expected that blood NK cells aren't currently used as a diagnostic test in women with PI, and measuring uterine NK cells will probably be a better option.

Conclusion

Our results revealed that despite the normal $CD3^+$ T cells, the rate of CD8 T cells and CD56 NK cells significantly decreased in women with infertility compared to the controls. There is insufficient evidence to recommend T- cell and NK cell testing of peripheral blood as a screening test in patients with PI.

Ethics

Ethics Committee Approval: Approval of the study protocol by the Ethics Committee of Najafabad University of Medical Sciences (IR.IAU.NAJAFABAD.REC.1399.119).
Informed Consent: Written informed consent was obtained from the patients.
Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: B.S., S.M., S.I., M.M., Design: B.S., S.M., S.I., M.M., Data Collection or Processing: B.S., S.M., S.I., M.M., Analysis or Interpretation: B.S., S.M., S.I., M.M., Literature Search: B.S., S.M., S.I., M.M., Writing: B.S., S.M., S.I., M.M.
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High expression of CD8 in the tumor microenvironment is associated with PD-1 expression and patient survival in high-grade serous ovarian cancer

Tümör mikroçevresinde CD8'in yüksek ekspresyonu, yüksek dereceli seröz over kanserinde PD-1 ekspresyonu ve hasta sağkalımı ile ilişkilidir

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Abstract

Objective: The current study assesses programmed death-1 (PD-1) receptor expression and CD3, CD4, and CD8 tumor-infiltrating lymphocytes (TILs) in high-grade serous ovarian cancer (HGSOC) and associates our results with neoadjuvant chemotherapy history and disease prognosis.

Materials and Methods: We included cases diagnosed with primary HGSOC with biopsy or surgical resection materials in this study. The immunoreactivity of CD3, CD4, CD8, and PD1 was assessed immunohistochemically in tumor tissue. We analyzed TILs in two predetermined groups of high and low TIL. The relationships between clinical characteristics, PD-1, and TIL were assessed. by the $\chi^{(2)}$ test or Fisher's Exact test. We used Kaplan-Meier survival analysis and Cox proportional hazards regression model to the connection between survival and the amounts of TIL, and PD1.

Results: Univariate analysis demonstrated that optimal debulking (p<0.001), early International Federation of Gynecology and Obstetrics stage (p=0.046), and higher scores of stromal CD8+ TIL expression (p=0.028) in tumor cells were all substantially correlated with longer disease-free survival (DFS), whereas the remaining variables analyzed, including PD-1 positivity, stromal CD3+, and CD4+ TILs, and intraepithelial CD3+, CD4+, and CD8+ TILs, were not correlated with DFS. Also, univariate analysis revealed that optimal debulking (p=0.010), and higher scores of stromal CD8+ TIL expression (p=0.021) in tumor cells were all substantially correlated with longer overall survival (OS).

Conclusion: Higher scores of stromal CD8+ TILs are substantially correlated with DFS and OS in univariate analyses, whereas scores of stromal CD3+ and CD4+ TILs, and intraepithelial CD3+, CD4+, and CD8+ TILs are not correlated with DFS and OS in both univariate and multivariate analyses. Also, we found a significant association between PD-1 positivity and the scores of stromal CD3+ TILs and intraepithelial CD8+ TILs. However, no remarkable relationship was revealed between PD-1 positivity and the survival of HGSOC cases.

Keywords: High-grade serous ovarian cancer, programmed death-1 receptor, tumor-infiltrating lymphocytes

PRECIS: High expression of CD8+ TILs in the tumor microenvironment is connected with PD-1 expression and longer patient survival in high-grade serous ovarian cancer.

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Öz

Amaç: Çalışmamızın amacı, yüksek dereceli seröz over kanserinde (HGSOC) programlanmış ölüm-1 (PD-1) reseptör ekspresyonunu ve CD3, CD4 ve CD8 tümör infiltre edici lenfositleri (TIL) değerlendirmek ve bulgularımızın neoadjuvan kemoterapi öyküsü ve hastalık prognozu ile ilişkisini incelemektir.

Gereç ve Yöntemler: Biyopsi veya cerrahi rezeksiyon materyalleri ile primer HGSOC tanısı alan olgular çalışmaya dahil edildi. CD3, CD4, CD8 ve PD1'in immünoreaktivitesi, tümör dokusunda immünohistokimyasal olarak değerlendirildi. TIL, önceden tanımlanmış iki grup olan düşük ve yüksek TIL grubunda analiz edildi. Klinik özellikler, PD-1 ve TIL arasındaki ilişkiler $\chi^{(2)}$ testi veya Fisher's Exact test ile değerlendirildi. TIL, PD1 ve hayatta kalma arasındaki ilişki için Kaplan-Meier hayatta kalma analizi ve Cox oransal hazard regresyon modeli kullanıldı.

Bulgular: Tek değişkenli analiz, tümör hücrelerinde optimal debulking (p<0,001), erken Uluslararası Jinekoloji ve Obstetrik Federasyonu evresi (p=0,046) ve daha yüksek stromal CD8+ TIL ekspresyonu skorlarının (p=0,028) tümünün daha uzun hastalıksız sağkalım (DFS) ile önemli ölçüde ilişkili olduğunu gösterdi; oysa ki kalan değişkenler, PD-1 pozitifliği, stromal CD3+ ve CD4+ TIL'ler ve intraepitelyal CD3+, CD4+ ve CD8+ TIL'ler dahil olmak üzere, analiz edildiğinde DFS ile korele değildi. Ayrıca, tek değişkenli analiz, tümör hücrelerinde optimal debulking (p=0,010) ve daha yüksek stromal CD8+ TIL ekspresyonu skorlarının (p=0,021) tümünün daha uzun genel sağkalım (OS) ile önemli ölçüde ilişkili olduğunu ortaya koydu.

Sonuç: Daha yüksek stromal CD8+ TIL skorları, tek değişkenli analizde DFS ve OS ile anlamlı şekilde ilişkiliyken, stromal CD3+ ve CD4+ TIL'lerin ve intraepitelyal CD3+, CD4+ ve CD8+ TIL'lerin skorları, hem tek değişkenli hem de çok değişkenli analizlerde DFS ve OS ile ilişkili değildi. Ayrıca, PD-1 pozitifliği ile stromal CD3+ TIL'lerin ve intraepitelyal CD8+ TIL'lerin skorları arasında anlamlı bir ilişki bulundu. Ancak, PD-1 pozitifliği ile HGSOC hastalarının sağkalımı arasında anlamlı bir ilişki gözlenmedi.

Anahtar Kelimeler: Yüksek dereceli seröz over kanseri, programlanmış ölüm-1 reseptörü, tümör infiltre edici lenfositler

Introduction

Globally, epithelial ovarian cancer is the third most frequent gynecologic cancer but has the highest death rate among gynecologic malignancies. Annually 313,000 women (3.4% of all cancer patients) are diagnosed with ovarian cancer worldwide and it is estimated to cause more than 200,000 deaths (4.7% of all cancer patients) occurred every year[1]. Among these cancers, high-grade serous ovarian cancer (HGSOC) constitutes 80-85% of these cancers and represents the highest mortality rate[2]. Due to the absence of effective screening methods and its non-specific early symptoms, most of the patients are diagnosed at advanced stages, with a five-year survival rate below 45%[3-5]. Various variables have been elucidated for the prognosis of HGSOC patients such as the stage at diagnosis, the extent of debulking surgery, and chemotherapy response[6]. Despite the considerable advances in complete debulking surgery, chemotherapeutic drugs, and targeted agents, survival ratios for ovarian cancer have unsatisfactorily improved over the past few decades and most patients have experienced drug resistance and cancer progression[7]. Thus far, no useful biochemical markers have been detected that might accurately predict the treatment response and survival of HGSOC. Therefore, there is an imperative requirement for superior plans for early recognition, prediction of prognosis, and efficient treatments to improve clinical consequences.

Adaptive and innate immune system cells perform a crucial role in eliminating cancer cells and have a significant impact on clinical outcomes in cancer patients. Cancer cells induce alterations in the immune context of the patient to regulate a supportive but definitely immunosuppressive tumor microenvironment (TME), producing neoantigens that interest various cells of the immune system[8]. Emerging studies indicate that ovarian cancer is frequently accompanied by a greatly systemic immunosuppressive TME[9,10]. This is the crucial element compromising the success rate of anticancer immunotherapy[10]. Patients who display a powerful immune response demonstrate a superior chemotherapy response and improved survival rates[9]. Tumor-infiltrating lymphocytes (TILs) are an essential constituent of the cellular immune system and crucial for cell-mediated anti-tumor immune responses[11]. There are various types of TILs with different functions. TILs consist of all lymphocytic cell populations that have left the vasculature and migrated to tumors. These cells are localized in the peritumoral space and the tumor islet (intraepithelial), and display an endogenous anti-cancer immune response[12]. TILs are identified by the cell surface expression of diverse molecular biomarkers such as CD3, CD4, and CD8, and demonstrate significantly different antitumor activities and spatial distribution among tumor areas[13]. After the recognition of specific antigens in MHC-I, cytotoxic CD8 T lymphocytes (immune system's classic killers) kill target cells by expressing cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor (TNF) and enzymes like perforin and granzyme-B. Conversely, CD4 T-cells are infrequently cytotoxic and instead promote other cells' recruitment and activation, including macrophages, B-cells, dendritic cells, and other T-cells[14]. TILs have been described in many solid tumors, and are considered to display a crucial role in mediating the chemotherapy response and improving survival in virtually entire solid tumor types, including ovarian cancer[12]. Ovarian carcinoma is an immunogenic disorder that is recognized and attacked by the immune system, and TILs are considered to recognize cancer cells to cause an immune response[15]. Numerous studies have concluded that the existence of TILs is significantly correlated with favorable prognoses in HGSOC cases[8,13]. However, there have also been some studies with conflicting results[9]. TILs might inhibit the immune response effectiveness due to adaptive immune resistance[16]. The inconsistency in results proposes that the prognostic value of TILs in patients with HGSOC remains controversial.

Measuring the levels of TILs extensively used the procedure to obtain detailed information on the interplay between the TME and the immune system. The current study assesss programmed death-1 (PD-1) expression and CD3+, CD4+, and CD8+ TIL in HGSOC and to associate our results with neoadjuvant chemotherapy (NACT) history and disease prognosis.

Materials and Methods

Patients

A total of 268 HGSOC patients were assessed and treated at the Kanuni Sultan Süleyman Training and Research Hospital, Department of Gynecologic Oncology, and partly Medipol University Department of Medical Oncology between February 2001 and April 2020, and 127 patients had adequate tumor samples and clinical data for analysis. We classified tumors histologically based on the criteria of the World Health Organization and staged based on the International Federation of Gynecology and Obstetrics (FIGO) system.

All patients with suspected advanced stage HGSOC were evaluated by a gynecologic oncologist before the beginning of treatment whether these cases were suitable for primary complete debulking surgery. Patients who had a low probability of achieving cytoreduction to <1 cm or had a high perioperative risk profile received NACT[17]. Maximal debulking surgery was defined as no visible disease remaining at the completion of the surgery. Optimal debulking surgery was defined as one or more tumor nodules <1 cm in maximal dimension remaining at the end of the surgical procedure. Suboptimal debulking surgery was described as any residual tumor nodule >10 mm in maximal dimension remaining at the end of the surgery[18]. All patients who experienced primary surgery and neoadjuvant and/or adjuvant platinum-based first-line chemotherapy were applied according to the stage. We obtained all the primary tumor tissue samples at the primary surgery time. The study was designed retrospectively and detailed clinical data of the cases were recorded from the patient's medical charts. The study was approved by the Medipol University Ethics Committee Resolution 10840098-604.01.01-E.17851, dated July 26, 2020.

Immunohistochemical Analysis of TIL and Scoring

Immunohistochemistry (IHC) was performed using primary antibodies against CD3+, CD4+, CD8+ TILs, and PD-1. Formalin-fixed, paraffin-embedded tissue sections (3 µm thick) were taken on 3-aminopropyltriethoxysilane coated glass slides. The sections were deparaffinized in xylene followed by hydration in graded ethanol. Histologic sections from tumors were stained with hematoxylin and eosin (H&E). For IHC representatives, prestained sections were selected, and 3-µm sections were obtained from paraffin-embedded tumors. CD3 rabbit monoclonal antibody (1:50 dilution, clone EP41, Biocare, Pacheco, USA), CD4 mouse monoclonal antibody (1:100 dilution, clone 4B12, Biocare, Pacheco, USA), CD8 mouse monoclonal antibody (1:100 dilution, clone C8/144B, Biocare, Pacheco, USA) and PD1 mouse monoclonal antibody (1:100 dilution, clone NAT105, Sigma-Aldrich, Germany) staining were performed according to the manufacturer's protocol (Figures 1-3).

The IHC slides were examined by two experienced pathologists under microscopy (Olympus) without any clinical data of the cases. Tumor cells, TILs, and PD-1 positivity were evaluated in H&E-stained sections.

The CD3, CD4, and CD8 protein staining was assessed based on the International TIL Working Group 2014 recommendations. Briefly, the assessment of TILs was carried out by manual visual evaluation of the percentage area covered by lymphocytes, after a standardized procedure[19]. Accordingly, the area within the tumor border is selected, the stromal and intra-tumoral compartments are defined, only mononuclear lymphocytic infiltrate areas are included, and the percentage area is evaluated at low (4x) and high (10x) magnification. Patients were identified to have a high expression of CD3, CD4, or CD8 TILs if these cases had ≥10% positive staining cells/HPF whereas low expression of TILs if these cases had <10% positive staining cells/HPF. We scored the stromal and intratumoral compartments separately. The stromal and intratumoral CD3, CD4, or CD8 TILs were classified into two groups for the analysis of low TIL (<10%) and high TIL (≥10%).

Tumor samples stained with anti- PD-1 were scored according to the intensity of cytoplasmic and/or membranous positivity as follows: 0 (no staining), 1 + (weak or equivocal staining), 2 +

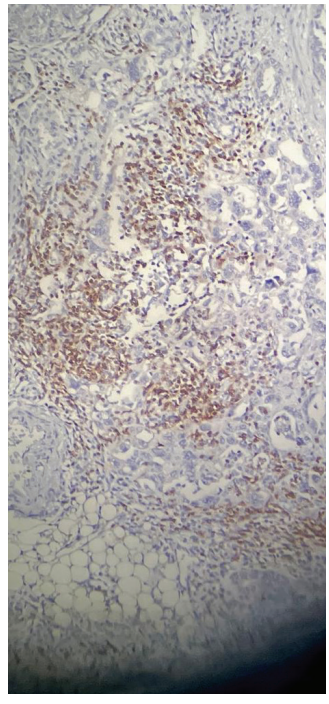

Figure 1. Stromal and intraepithelial CD3+ tumor-infiltrating lymphocytes (IHC, x200)

IHC: Immunohistochemistry

(moderate staining), or 3 + (strong staining). Tumor cells and micro-environment were considered as positive for staining if they had more than 5% staining[20].

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., Armonk, NY). We used descriptive statistics to summarize baseline features. We used Fisher's Exact test and Pearson chi-square (χ^2) test to determine the relationship between clinicopathological factors and TIL status in patients with serous ovarian cancer. We made survival analysis via Kaplan-Meier analysis and comparisons via the Log-Rank test. We defined disease-free survival (DFS) as the date from diagnosis or curative surgery to recurrence or disease progression or the time of death or loss in the follow-up. We described overall survival (OS) as the date from diagnosis to the time of the patient's death or loss in the follow-up. Univariate analysis was performed to evaluate the significance of clinicopathological characteristics as prognostic factors. Subsequently, we performed a multivariate analysis with the Cox proportional hazards model to detect the independent prognostic factors for both DFS and OS. We used multivariate p-values to describe the independence of these factors. Data were presented as mean ± standard deviation, median (minimum-maximum), 95% confidence interval, and percentage (%) where appropriate. P-values <0.05 were considered statistically significant.

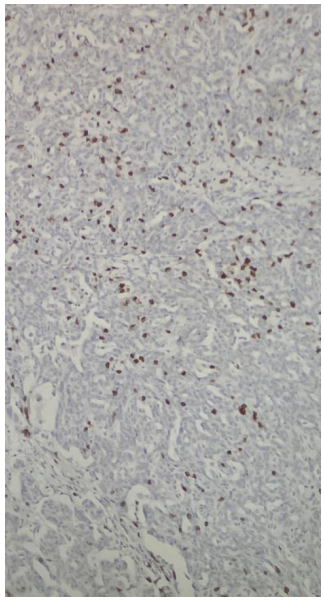

Figure 2. Intraepithelial CD4+ tumor-infiltrating lymphocytes (IHC, x200)

IHC: Immunohistochemistry

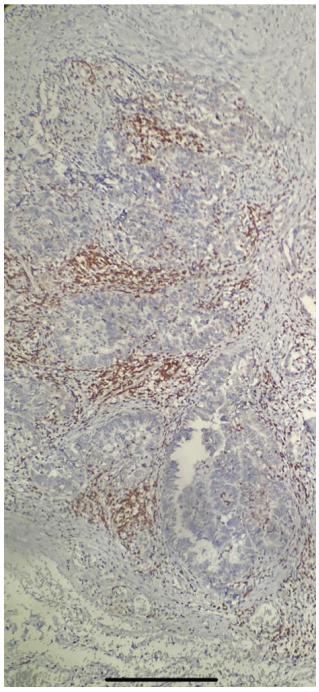

Figure 3. Stromal CD8+ tumor-infiltrating lymphocytes (IHC, x200)

IHC: Immunohistochemistry

Results

The demographic and basic data of the participants are listed in Table 1. Of the study cohort of 127 cases, the median age at diagnosis was 55 years with a range approximately 26-83 years. Most cases (n=89, 71.2%) presented FIGO stage III or IV. Within the 127 cases, 77.4% (n=96) n of them received NACT. Of these, 89 cases presented with advanced stage of disease, and 7 patients had a high perioperative risk profile. Maximal debulking was achieved in 75.6% (n=93), and optimal debulking was achieved in 16.3% (n=20) patients. The median OS was 37.5 months (ranging between 3.7 and 94.9 months) for the entire study population.

The relationship between clinicopathological factors and PD-1 expression in patients with serous ovarian cancer is presented in Table 2. PD-1 was detected in intraepithelial and stromal TILs in 66.9% (n=85) of cases. PD-1 positive and PD-1 negative groups were similar regarding age, receiving NAC, surgery type, tumor grade, and FIGO stage at diagnosis. The PD-1-positive group had significantly higher numbers of stromal CD3+ TILs and intraepithelial CD8+ TILs than the PD-1-negative group (p=0.043, p=0.003, respectively). However, we found no association between PD-1 positivity and the scores of stromal CD4+ (p=0.073) and CD8+ TILs, and intraepithelial CD3+ and CD4+ TILs.

We summarize univariate and multivariate analyses of risk factors for DFS in cases of serous ovarian cancer in Table 3. Univariate analysis demonstrated that optimal debulking (p<0.001), early FIGO stage (p=0.046), and higher scores of stromal CD8+ TIL expression (p=0.028) in tumor cells were all substantially correlated with longer DFS, whereas the remaining variables analyzed, including PD-1 positivity, stromal CD3+, and CD4+ TILs, and intraepithelial CD3+, CD4+, and CD8+

TILs, were not correlated with DFS. Multivariate analysis showed that optimal debulking (p<0.001) was an independent prognostic variable for DFS, whereas FIGO stage and higher scores of stromal CD8+ TILs (p=0.055) were unsubstantial predictive factors for DFS in multivariate analysis. Although slightly far from statistical significance at a 0.05 threshold, a comparable tendency was detected in the DFS analysis; cases with CD8+ tumors seemed to have better DFS than those with CD8-negative tumors (p=0.055).

Univariate and multivariate analyses of risk factors for OS in patients with serous ovarian cancer are shown in Table 4. Univariate analysis revealed that optimal debulking (p=0.010), and higher scores of stromal CD8+ TIL expression (p=0.021) in tumor cells were all substantially correlated with longer OS, whereas the remaining variables analyzed, including FIGO stage, PD-1 positivity, stromal CD3+, and CD4+ TILs, and intraepithelial CD3+, CD4+, and CD8+ TILs, were not associated with OS. Multivariate analysis showed that only optimal debulking (p=0.026) was an independent prognostic variable for OS, whereas a higher score of stromal CD8+ TIL expression was not a significant predictive factor for OS in multivariate analysis.

Table 1. Demographic and basic patient information

Characteristics (units)	Average (range) n=127
Age (years)	55 (26-83)
Preop plasma CA125 (IU/L)	210 (4-9777)
FIGO	n (%)
Stage I/II	36 (28.8)
Stage III/IV	89 (71.2)
Surgery type	n (%)
Maximal debulking	93 (75.6)
Optimal debulking	20 (16.3)
Suboptimal debulking	8 (6.5)
Inoperable	2 (1.6)
Neoadjuvant therapy	n (%)
Absent	28 (22.6)
Present	96 (77.4)
Tumor grade	n (%)
1	10 (8)
2	29 (23.2)
3	86 (68.8)
PD-1 expression	n (%)
Negative	42 (33.1)
Positive	85 (66.9)
Median OS (months)	37.5 (3.73-94.93)

OS: Overall survival, FIGO: International Federation of Gynecology and Obstetrics

Figures 4 and 5 demonstrate the Kaplan-Meier curves of median DFS and median OS regarding the scores of stromal CD8+ TILs, respectively. Cases in the stromal CD8-high TIL group had substantially longer DFS (28.3 months) and OS (83.8 months) than those in the CD8-low TIL group (15.3 months and 65.7 months, p=0.028 and p=0.021, respectively).

Discussion

In this study, we investigated the effects of CD3, CD4, and CD8 T-cell status on survival in patients with HGSOC. Also, we clarified PD-L1 expression and TIL infiltration in HGSOC cases. As evidenced by our study, stromal CD8+ TILs alone were indicative of improved survival in patients with HGSOC. However, the presence of intraepithelial CD3+, CD4+, and CD8+ TILs, and stromal CD3+ and CD4+ TILs were not associated with the prognosis of ovarian cancer.

The heterogeneity of stromal TILs with cancer cell proliferation, invasion, and matrix remodeling that drive carcinogenesis ending in diverse survival periods generates a specific TME. Cancer cells interact with their TME both to induce tumorigenic inflammation and suppress T-cell activation[21]. Transformed tumor cells, as a source of neoantigens or tumor-related antigens, might stimulate the immune response, eventually ending in tumor cell elimination by TILs[22]. The density, subtype, and location of TILs are determining factors of the prognostic importance of TILs in ovarian malignancy[23]. Considering the beneficial treatments, particularly immunotherapy modalities to TILs, it must assess the presence and density of TILs and realize tumor-immune system interactions in ovarian malignancy[24]. Various studies have reported explored the prognostic significance of TILs in ovarian cancer. Despite these studies using biomarkers to clarify the diverse subtypes of TILs that impact prognosis and survival, the outcomes were indicated have still been inconsistent[25]. The possible reasons for inconsistent findings might involve sample size, tumor heterogeneity, tumor type, clinical stage, variations in the regions of the study cohort, and the technique for specimen processing[15].

CD3 antigen, known as pan T-cell marker, is a receptor glycoprotein that exists in mature lymphocytes. Zhang et al.[26] reported that the increased expression of VEGF was related to the lack of CD3+ TILs, and thus early recurrence and short survival. They found that the CD3+ TILs associated with improved OS and PFS in ovarian cancer patients[26]. However, in our study, similar to the study of Sato et al.[27] and Lo et al.[28], neither stromal nor intraepithelial CD3+ TILs alone were connected with the survival of patients with ovarian cancer.

CD4+ TILs exhibit a different variety of antitumor immune responses. CD4+ T-helper 1 (Th1) cells secreting cytokines, including TNF and IFN-γ might efficiently suppress angiogenesis and promote the proliferation and activation of CD8+ TILs. In contrast, CD4+ T-regulatory (Treg) cells display tumor-promoting activity by inhibiting Th1 cell function and inhibiting autoimmunity development[8]. Pinto et al.[9] found

Table 2. Relationship between clinicopathological factors and PD-1 in patients with serous ovarian cancer

Factors	n (%)	PD-1 positive n (%)	PD-1 negative n (%)	p-value
All patient	127 (100)	85 (66.9)	42 (33.1)	
Age (year)				
<50	38 (29.9)	29 (76.3)	9 (23.7)	0.156
>50	89 (70.1)	56 (62.9)	33 (37.1)	
NAC				
Absence	96 (77.4)	64 (66.7)	32 (33.3)	1.0
Presence	28 (22.6)	19 (67.9)	9 (32.1)	
Surgery type				
Maximal debulking	93 (75.6)	65 (69.9)	28 (30.1)	0.287
Optimal debulking	20 (16.3)	13 (65)	7 (35)	
Suboptimal debulking	8 (6.5)	3 (37.5)	5 (62.5)	
Inoperable	2 (1.6)	1 (50)	1 (50)	
Tumor grade				
1	10 (8)	8 (80)	2 (20)	0.621
2	29 (23.2)	20 (69)	9 (31)	
3	86 (68.8)	56 (65.1)	30 (34.9)	
FIGO stage				
I-II	36 (28.8)	26 (72.2)	10 (27.8)	0.531
III-IV	89 (71.2)	58 (65.2)	31 (34.8)	
Stromal CD3				
Low TILs	39 (30.7)	21 (53.8)	18 (46.2)	**0.043**
High TILs	88 (69.3)	64 (72.7)	24 (27.3)	
Stromal CD4				
Low TILs	107 (84.3)	68 (63.6)	39 (36.4)	0.073
High TILs	20 (15.7)	17 (85)	3 (15)	
Stromal CD8				
Low TILs	80 (63)	57 (71.3)	23 (28.8)	0.241
High TILs	47 (37)	28 (59.6)	19 (40.4)	
Intraepithelial CD3				
Low TILs	45 (35.4)	27 (60)	18 (40)	0.241
High TILs	82 (64.6)	58 (70.7)	24 (29.3)	
Intraepithelial CD4				
Low TILs	104 (81.9)	69 (66.3)	35 (33.7)	1.000
High TILs	23 (18.1)	16 (69.6)	7 (30.4)	
Intraepithelial CD8				
Low TILs	35 (27.6)	16 (45.7)	19 (54.3)	**0.003**
High TILs	92 (72.4)	69 (75)	23 (25)	

PD-1: Programmed death-1, NAC: Neoadjuvant chemotherapy, FIGO: International Federation of Gynecology and Obstetrics, TILs: Tumor-infiltrating lymphocytes

Table 3. Univariate and multivariate analysis of risk factors for DFS in patients with serous ovarian cancer

Factors	Median DFS time (months)	Univariate p-value	Multivariate p-value	HR (95% CI)
Age (year)				
<50	23.0	0.423		
>50	17.0			
NAC				
Absence	16.0	0.08		
Presence	21.0			
Surgery type				
Maximal debulking	28.3	**<0.001**	**<0.001**	**1.69 (1.23-2.33)**
Optimal debulking	15.7			
Suboptimal debulking	11.4			
Inoperable	6.9			
Tumor grade				
1	NR	0.055		
2	25.6			
3	16.9			
FIGO stage				
I-II	52.4	**0.046**	0.160	0.60 (0.29-1.22)
III-IV	16.9			
PD-1 expression				
Negative	23.5	0.64		
Positive	18.4			
Stromal CD3				
Low TILs	16.0	0.621	0.136	0.52 (0.22-1.22)
High TILs	18.8			
Stromal CD4				
Low TILs	20.3	0.763	0.316	1.61 (0.63-4.09)
High TILs	15.7			
Stromal CD8				
Low TILs	15.3	**0.028**	0.055	**2.23 (0.98-5.08)**
High TILs	28.3			
Intraepithelial CD3				
Low TILs	23.5	0.705	0.747	1.14 (0.50-2.56)
High TILs	16.0			
Intraepithelial CD4				
Low TILs	16.9	0.216	0.175	0.50 (0.19-1.35)
High TILs	32.8			
Intraepithelial CD8				
Low TILs	23.5	0.665	0.867	0.93 (0.39-2.18)
High TILs	16.0			

PD-1: Programmed death-1, NAC: Neoadjuvant chemotherapy, FIGO: International Federation of Gynecology and Obstetrics, TILs: Tumor-infiltrating lymphocytes, CI: Confidence interval, DFS: Disease-free survival, HR: Hazard ratio

Table 4. Univariate and multivariate analysis of risk factors for OS in patients with serous ovarian cancer

Factors	Median OS time (months)	Univariate p-value	Multivariate p-value	HR (95% CI)
Age (year)				
<50	44.9	0.799		
>50	47.7			
NAC				
Absence	50.5	0.111		
Presence	37.0			
Surgery type				
Maximal debulking	NR	**0.010**	**0.026**	**1.56 (1.05-2.33)**
Optimal debulking	48.9			
Suboptimal debulking	35.5			
Inoperable	36.9			
Tumor grade				
1	78.7	0.076		
2	50.5			
3	39.9			
FIGO stage				
I-II	50.5	0.642	0.517	0.75 (0.27-1.91)
III-IV	45.0			
PD-1 expression				
Negative	45.6	0.870		
Positive	47.7			
Stromal CD3				
Low TILs	NR	0.385	0.817	0.87 (0.29-2.61)
High TILs	60.8			
Stromal CD4				
Low TILs	72.6	0.776	0.724	0.75 (0.16-3.53)
High TILs	NR			
Stromal CD8				
Low TILs	65.7	**0.021**	0.211	1.96 (0.68-5.66)
High TILs	83.8			
Intraepithelial CD3				
Low TILs	72.66	0.309	0.719	1.23 (0.39-3.84)
High TILs	60.8			
Intraepithelial CD4				
Low TILs	72.6	0.594	0.742	0.81 (0.24-2.71)
High TILs	64.4			
Intraepithelial CD8				
Low TILs	72.6	0.365	0.794	1.17 (0.35-3.85)
High TILs	60.8			

PD-1: Programmed death-1, NAC: Neoadjuvant chemotherapy, FIGO: International Federation of Gynecology and Obstetrics, TILs: Tumor-infiltrating lymphocytes, CI: Confidence interval, DFS: Disease-free survival, HR: Hazard ratio, OS: Overall survival

that CD4+ TILs are the early predictor factors of OS and PFS in patients with HGSOC. However, a recent study conducted in Turkey concluded that the existence of CD4+ TILs was more frequent in advanced stages ovarian cancer patients, and no significant relationship was found between this subtype of TILs and survival[21]. Likewise, we observed no significant association between higher levels of stomal and intraepithelial CD4+ TILs and a favorable prognosis. To demonstrate CD4+ TIL biology in connection with tumor progression in ovarian cancer patients, additional analysis on the functional subtypes of CD4+ TILs, including Th1 and Treg, is required in forthcoming research[8].

CD8+ TILs perform a central role in immunity to cancer through their capacity to directly kill tumor cells after recognition of specific antigens in MHC-I molecules[14]. We demonstrated

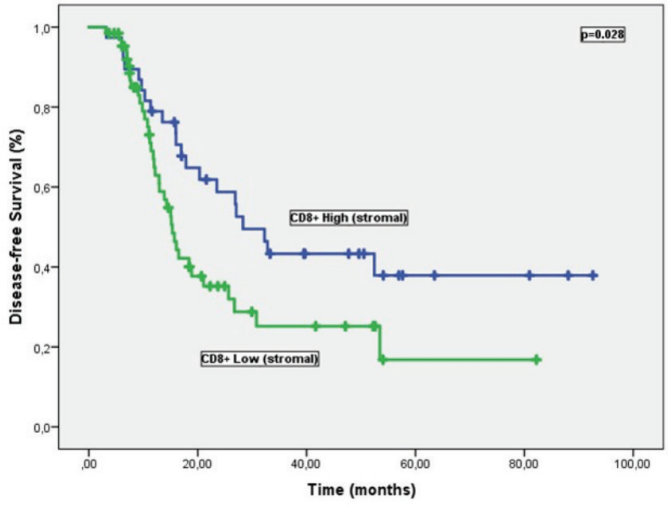

Figure 4. Analysis of disease-free survival regarding stromal CD8 positivity status

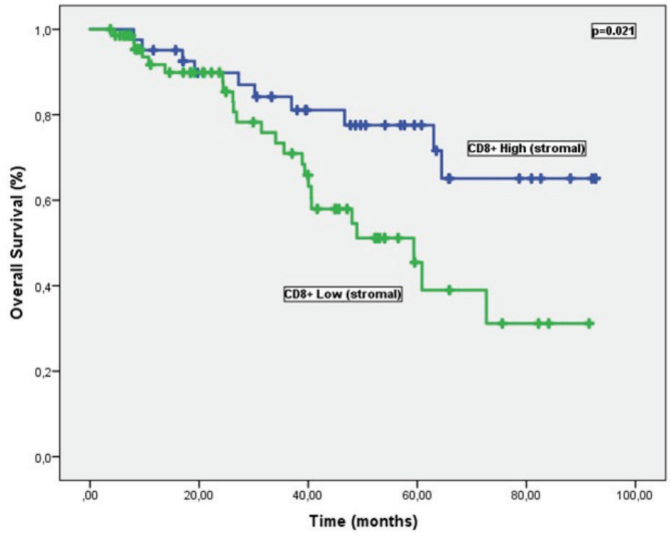

Figure 5. Analysis of overall survival regarding stromal CD8 positivity status

that the stromal CD8+ TIL infiltration was correlated with better DFS and OS in HGSOC patients in univariate analysis. Nonetheless, following adjusting the confounding variables in the multivariate analysis, we did not verify its significance as an outcome predictor of DFS and OS. Even though slightly far from statistical signification at a 0.05 threshold, HGSOC patients with CD8+ TILs seemed to have better DFS than those with CD8-negative cases. Also, we found that intraepithelial CD8+ TILs did not correlate with improved DFS and OS in our cohort of HGSOC patients. In a meta-analysis, Li et al.[15] investigated whether the specific location of CD8+ TILs within the tumor mass is crucial for the prognostic impact on ovarian cancer. They indicated that intraepithelial TILs are related to a favorable prognosis in ovarian cancer, underlining the significance of assessing the localization of TILs within the TME[15]. However, Hao et al.[8] confirmed that both intraepithelial and stromal CD8+ TILs are positively correlated with OS and progression-free survival (PFS) cases with HGSOC. A recently published study on the Turkish population indicated that CD8+ TIL infiltration was connected with advanced stage and worse prognosis in ovarian cancer[21]. Several experiments have been conducted to clarify the mechanisms that provide the location and infiltration of TILs into tumor islets in ovarian cancer[23]. However, to date, due to the immune system plasticity and the tumor genome complexity, the precise mechanism remains unclear. Schietinger et al.[29] reported that CD8+ TILs might be involved in the destruction of stromal components such as endothelial cells, causing tumor necrosis. Also, this might end in impacts against antigen-negative cancer cells in tumors. Moreover, stromal cells might perform a function in antigen presentation to improve T-cell activity against cancer cells[29]. Therefore, the stromal accumulation of CD8+ TILs without direct interplay with tumor cells is critical for cancer removal[25]. Comprehension of the interplay between ovarian cancer and the immune system might be a significant stage toward detecting prognostic gene markers, overcoming drug resistance and providing longer life expectancy of patients with ovarian cancer[24].

The PD-1 receptor is expressed by activated T-cells and has two recognized ligands, of which PD-L1 can be expressed by tumor cells and adjacent immune cells in various solid tumors. The binding of PD-L1 to PD-1 inhibits T-cell receptor signaling, resulting in decreased T-cell proliferation and enhanced vulnerability to apoptosis[30]. The PD-1/PD-L1 pathway is supposed to be the main regulator of tumor-induced immune suppression[11]. Anti-PD-1 immunotherapy promotes persisted T-cell activity to prevent apoptosis of these cells and is effective in a broad variety of malignancies[31]. However, Hao et al.[8] and Lo et al.[28] indicated that overexpression of PD-1+ TILs was not related to the survival benefit of HGSOC cases. Hao et al.[8] also stated that the association between survival outcomes and PD-1+ TILs still warrants additional research because of the comprehensively increasing immunotherapy

implications[28]. Because few studies have been published regarding the association between TILs and PD-1 positivity in ovarian cancer[30,32-34], we assessed the correlations between PD-1 positivity and the scores of TILs. PD-1-positive cases include substantially greater numbers of stromal CD3+ TILs and intraepithelial CD8+ TILs than in the PD-1-negative cases, whereas no significant associations were observed between PD1 positivity and scores of intraepithelial CD3+ and CD8+ TILs, and stromal CD4+ and CD8+ TILs. We also showed no significant relationship between PD-1 expression and survival outcomes in TILs. Thus, the efficiency of PD-1/PD-L1 blockade in ovarian cancer is comparatively lower than that in melanoma, gastric cancer, and cervical cancer. A possible reason for the comparatively low efficiency of PD-1/PD-L1 blockade in ovarian cancer might be that the cases included in these studies lack existing CD8+ TILs and PD-L1 expression in tumors. Assessment of CD8+ TIL count and PD-L1 expression might be beneficial in the stratification of HGSOC cases for PD-1/PD-L1 blockade therapy.

Study Limitations

The main limitations of this study are the retrospective nature, comparatively small number of cases, and a comparatively short period of follow-up. The main study strength is that few studies in the literature investigate the clinicopathologic and molecular features of TILs in tumor cells in HGSOC in the Turkish population.

Conclusion

This study revealed that higher scores of stromal CD8+ TILs are substantially correlated with DFS and OS in univariate analyzes, whereas scores of intraepithelial CD3+, CD4+, and CD8+ TILs, and stromal CD3+ and CD4+ TILs are not correlated with DFS and OS in both univariate and multivariate analyses. Also, we found a significant association between PD-1 positivity and the scores of stromal CD3+ TILs and intraepithelial CD8+ TILs. However, no significant association was detected between PD-1 positivity and the survival of HGSOC patients.

Ethics

Ethics Committee Approval: The study was approved by the Medipol University ethics committee resolution 10840098-604.01.01-E.17851, dated July 26, 2020.
Informed Consent: Retrospective study.
Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: F.Ö., Ö.F.Ö., Ö.A., S.A., Concept: F.Ö., S.C.O., Ö.F.Ö., Ö.A., E.Y., S.A., M.K., N.A.S., A.K.K., Design: F.Ö., S.C.O., Ö.F.Ö., Ö.A., E.Y., S.A., M.K., N.A.S., A.K.K., Data Collection or Processing: F.Ö., Ö.F.Ö., Ö.A., S.A., Analysis or Interpretation: F.Ö., S.C.O., Ö.F.Ö., E.Y., Literature Search: F.Ö., S.C.O., Ö.F.Ö., E.Y., Writing: F.Ö., S.C.O., Ö.F.Ö., Critical Review: S.C.O., Ö.F.Ö.

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