E-ISSN : 2149-9330

TURKISH JOURNAL OF OBSTETRICS AND GYNECOLOGY

December 2025 Volume: 22 Issue: 4 www.tjoddergisi.org

TURKISH SOCIETY OF OBSTETRICS AND GYNECOLOGY

galenos yayınevi

TURKISH JOURNAL OF OBSTETRICS AND GYNECOLOGY

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Please refer to the journal's webpage **(https://tjoddergisi.org/)** for "Aims and Scope", "Instructions to Authors" and "Peer-Review and Ethics".

The editorial and publication process of the Turkish Journal of Obstetrics and Gynecology are shaped in accordance with the guidelines of ICMJE, WAME, CSE, COPE, EASE, and NISO. The journal is in conformity with the Principles of Transparency and Best Practice in Scholarly Publishing.

Turkish Journal of Obstetrics and Gynecology is indexed in **PubMed, PubMed Central (PMC), Web of Science-Emerging Sources Citation Index (ESCI), Tübitak/Ulakbim Turkish Medical Database, EBSCO, Embase, Scopus, ProQuest, British Library, Gale, CINAHL, Turk Medline, J-Gate, IdealOnline, CNKI, Hinari, GOALI, ARDI, OARE, AGORA** and **Turkiye Citation Index**.

The journal is published electronically.

Owner: İsmail Mete İtil on behalf of the Turkish Society of Obstetrics and Gynecology

Responsible Manager: Ercan Yılmaz

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Phone: +90 (530) 177 30 97 / +90 (539) 307 32 03 **E-mail:** info@galenos.com.tr/yayin@galenos.com.tr **Web:** www.galenos.com.tr
Publisher Certificate Number:14521

Online Publication Date: December 2025 **E-ISSN:** 2149-9330
International scientific journal published quarterly.

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TURKISH JOURNAL OF OBSTETRICS AND GYNECOLOGY

LETTER FROM THE PRESIDENT

Dear TJOD family,

As we bid farewell to 2025 and prepare to welcome 2026, I am pleased to meet you once again with the December issue of our scientific journal, which has achieved international success.

The Turkish Society of Obstetrics and Gynecology continues to remain on the agenda not only with our international journal but also with the many courses, symposia, and training programs it has organized and aims to organize. In particular, some statements made in September regarding the use of paracetamol in pregnant women, a topic of international debate, caused serious concern in our country as well. With the "TJOD Statement" we issued on this matter, we successfully put an end to the discussions. Once again, on the national platform, we held the final Resident School of 2025 in Malatya, after organizing many such programs in various provinces across our country. The "Course on Gynecological Ultrasonography and Management of Obstetric Anal Sphincter Injury" was conducted quite successfully with both its theoretical and practical components. I would also like to inform you that the next Resident Course is planned to take place in Istanbul in the early months of 2026 as the "Gynecological Laparoscopy" course.

Recognized by the International Federation of Obstetrics and Gynecology (FIGO) as the only officially acknowledged obstetrics and gynecology association in Türkiye, TJOD will continue, as it has in the past and still does today, to carry out work befitting its name and scientific stature.

Dear colleagues, I extend my wishes that 2026 brings peace, happiness, tranquility, and health to our country and the entire world, and I wish you a happy new year.

Best Regards
Ismail Mete Itil, Prof. MD.
President of TJOD

TURKISH JOURNAL OF OBSTETRICS AND GYNECOLOGY

EDITORIAL

Dear Colleagues,

We greet you with the joy of once again being before our scientific community with the final month of 2025 and the last issue of our journal. In our December issue, we have six scientific research articles.

We would like to state that we have a very large working team involved in the preparation of our journal. As a result of the devoted efforts, the meticulously processed articles are elevated to the level befitting our esteemed colleagues.

We would like to emphasize that in 2026, we will continue to do our utmost to work harder, produce more, and raise our scientific journal to the highest international standing.

With our wishes for 2026 to bring love, success, peace, and health.

Ercan Yilmaz, Prof. MD.
Fatih Sendag, Prof. MD.

Clinical Investigation / Araştırma

DOI: 10.4274/tjod.galenos.2025.88949

Turk J Obstet Gynecol 2025;22(4):287-91

The impact of human papillomavirus positivity and genotype on sexual dysfunction and psychosexual stress

İnsan papilloma virüs pozitifliği ve genotipinin cinsel işlev bozukluğu ve psikoseksüel stres üzerine etkisi

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Abstract

Objective: This study aimed to investigate the relationship between human papillomavirus (HPV) positivity, genotype, and female sexual dysfunction, particularly anorgasmia and psychosexual stress, among women participating in a cervical cancer screening program. It also examined whether HPV infection or genotype independently contributes to sexual dysfunction after adjusting for sociodemographic and reproductive factors.

Materials and Methods: This prospective, cross-sectional study included 1,353 sexually active women aged 25-65 years who underwent HPV testing at Antalya City Hospital between May and September 2025. Participants completed validated questionnaires including the Female Sexual Function Index, Arizona Sexual Experiences Scale, Beck Depression Inventory, and Beck Anxiety Inventory. Sociodemographic, reproductive, and clinical characteristics were recorded, and HPV genotyping was performed using polymerase chain reaction-based assays.

Results: Anorgasmia was identified in 31.5% of participants (n=427). It was significantly more common among unemployed women (84.1% vs. 71.6%; odds ratio =2.09, 95% confidence interval: 1.56-2.82; p=0.0001). Higher gravidity, parity, number of living children, and elevated vaginal pH were all associated with anorgasmia ($p<0.05$). No significant association was found between HPV positivity or genotype and anorgasmia ($p>0.05$).

Conclusion: Anorgasmia is primarily influenced by sociodemographic and reproductive factors, such as occupation, education level, parity, and vaginal environment, rather than HPV infection or genotype. These findings emphasize the importance of biopsychosocial and culturally sensitive approaches in evaluating and managing women's sexual health.

Keywords: Anorgasmia, papillomavirus infections, sexual dysfunction, physiological

PRECIS: Sociodemographic and reproductive factors, rather than HPV infection or genotype, were found to significantly influence anorgasmia and psychosexual stress among Turkish women undergoing cervical cancer screening in Antalya.

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Received/Geliş Tarihi: 20.10.2025 **Accepted/Kabul Tarihi:** 24.10.2025 **Epub:** 27.10.2025 **Publication Date/Yayınlanma Tarihi:** 02.12.2025

Cite this article as: Karadağ C, Gül Kara SM, Güner G, Dağdeviren E, Pota A, Tercan E, et al. The impact of human papillomavirus positivity and genotype on sexual dysfunction and psychosexual stress.Turk J Obstet Gynecol. 2025;22(4):287-91

Öz

Amaç: Bu çalışma, serviks kanseri tarama programına katılan kadınlarda insan papilloma virüsü (HPV) pozitifliği, genotipi ve kadın cinsel işlev bozukluğu özellikle anorgazmi ve psikoseksüel stres arasındaki ilişkiyi araştırmayı amaçladı. Ayrıca HPV enfeksiyonu veya genotipinin, sosyodemografik ve üreme faktörlerinden bağımsız olarak cinsel işlev bozukluğuna katkıda bulunup bulunmadığı incelendi.

Gereç ve Yöntemler: Bu prospektif, kesitsel çalışma, Mayıs-Eylül 2025 tarihleri arasında Antalya Şehir Hastanesi'nde HPV testi yapılan, 25-65 yaş arası 1.353 cinsel olarak aktif kadını içermektedir. Katılımcılar, Kadın Cinsel İşlev İndeksi, Arizona Cinsel Deneyimler Ölçeği, Beck Depresyon Envanteri ve Beck Anksiyete Envanteri gibi doğrulanmış anketleri doldurmuştur. Sosyodemografik, üreme ve klinik özellikler kaydedilmiş, HPV genotiplemesi ise polimeraz zincir reaksiyonu temelli testlerle yapılmıştır.

Bulgular: Katılımcıların %31,5'inde (n=427) anorgazmi tespit edilmiştir. Anorgazmi, çalışmayan kadınlarda anlamlı derecede daha yaygındır (%84,1'e karşı %71,6; risk oranı =2,09, %95 güven aralığı: 1,56-2,82; p=0,0001). Daha yüksek gebelik sayısı, doğum sayısı, yaşayan çocuk sayısı ve artmış vajinal pH değerleri anorgazmi ile anlamlı şekilde ilişkili bulunmuştur (p<0,05). HPV pozitifliği veya genotipi ile anorgazmi arasında anlamlı bir ilişki saptanmamıştır (p>0,05).

Sonuç: Anorgazmi, HPV enfeksiyonu veya genotipinden ziyade sosyodemografik ve üreme faktörleri özellikle meslek, eğitim düzeyi, doğurganlık ve vajinal ortam ile ilişkilidir. Bu bulgular, kadın cinsel sağlığının değerlendirilmesi ve yönetiminde biyopsikososyal ve kültürel açıdan duyarlı yaklaşımların önemini vurgulamaktadır.

Anahtar Kelimeler: Anorgazmi, papillomavirüs enfeksiyonları, cinsel işlev bozukluğu, fizyolojik

Introduction

Human papillomavirus (HPV) infection is the most common sexually transmitted disease worldwide and represents a major global public health concern due to its strong oncogenic potential[1,2]. Persistent HPV infection is strongly associated with the development of cervical intraepithelial neoplasia and cervical cancer, as well as other anogenital and oropharyngeal malignancies. Sexual dysfunction remains a widespread issue among women worldwide[2,3]. Beyond its oncological implications, HPV positivity may affect women's sexual and psychosocial well-being, an area that has been relatively understudied[4].

Previous research has demonstrated that HPV infection can negatively impact female sexual function. In a study conducted in the Turkish population, Mercan et al.[5] reported that women diagnosed with HPV infection experienced higher rates of sexual dysfunction, suggesting that the psychological burden of HPV positivity contributes to disturbances in sexual health. Similarly, Aker et al.[6] found that both HPV diagnosis and abnormal cervical cytology results were significantly associated with increased sexual dysfunction and heightened anxiety, underscoring the interplay between somatic and psychological factors in affected women.

In addition to biological consequences, HPV infection may trigger psychosexual stress through mechanisms such as fear of cancer, anxiety about transmission to partners, and concerns regarding stigma and body image[4,6]. These psychosocial responses can impair sexual satisfaction, reduce intimacy, and contribute to long-term sexual dysfunction. While international studies have identified associations between HPV infection, anxiety, and impaired quality of life, data from the Turkish context remain limited[5,6]. Considering cultural norms, sociopsychological dynamics, and healthcare access differences, further exploration of this topic is warranted.

Therefore, this study aims to evaluate the impact of HPV positivity and HPV genotype on female sexual dysfunction and psychosexual stress in women participating in cervical cancer screening programs. By integrating validated sexual function and psychometric assessment tools [Female Sexual Function Index (FSFI), Arizona Sexual Experiences Scale (ASEX), Beck Depression Inventory (BDI), Beck Anxiety Inventory (BAI)], this research seeks to provide evidence that may inform patient counseling, psychosocial support, and tailored management strategies in gynecologic practice.

Materials and Methods

This prospective, descriptive, and cross-sectional clinical study was conducted at the Gynecology Outpatient Clinics of Antalya City Hospital between May 2025 and September 2025. The study was approved by Antalya City Hospital Ethics Committee and was performed in accordance with the ethical standards described in the 1975 Declaration of Helsinki, as revised in 2000 (approval no: 2025-134, date: 28.05.2025), and all procedures were carried out in accordance with the ethical standards outlined in the Declaration of Helsinki (revised 2000). Written informed consent was obtained from all participants prior to enrollment.

A total of 1,353 sexually active women aged between 25 and 65 years who underwent HPV testing were included in the study. Participants were divided into two groups according to HPV status: women with positive HPV test results (any genotype) and age-matched women with negative test results. Women who were not sexually active, those with a previous history of gynecologic malignancy, pelvic surgery, or radiotherapy, those with severe psychiatric disorders, and women with incomplete questionnaire data were excluded from the study.

A priori power analysis was performed to estimate the minimum sample size required for adequate statistical power. For a correlation coefficient of r=0.4, with 95% power and an alpha level of 0.05, a minimum of 124 participants (62 HPV-positive and 62 HPV-negative) was deemed sufficient. However, to increase the robustness and generalizability of the findings, all eligible women who met the inclusion criteria, during the

study period were recruited, resulting in a final sample of 1,353 participants.

After obtaining consent, participants underwent a structured face-to-face interview conducted by trained gynecology residents in a private setting. Sociodemographic variables, including age, marital status, education level, and occupation, as well as obstetric and gynecologic history such as gravidity, parity, mode of delivery, and contraceptive methods, were recorded. Lifestyle factors, including smoking habits and the frequency of sexual intercourse, were also documented.

Sexual function was assessed using the Turkish-validated versions of two standardized instruments: the FSFI, a 19-item scale evaluating six domains (desire, arousal, lubrication, orgasm, satisfaction, and pain), and the ASEX, a 5-item questionnaire assessing sexual drive, arousal, orgasmic ability, and satisfaction. Psychosexual stress was evaluated using the BDI and the BAI, both of which are widely validated self-report tools measuring the severity of depressive and anxiety symptoms, respectively. All questionnaires were self-administered under supervision to ensure completeness and accuracy.

Cervical samples were obtained from all participants using standard cytobrush techniques. HPV DNA detection and genotyping were performed using polymerase chain reaction-based molecular assays capable of identifying both high-risk and low-risk HPV genotypes. Vaginal pH levels were measured immediately after sample collection using sterile pH indicator strips.

Statistical Analyses

Statistical analyses were performed using IBM SPSS Statistics version 25.0 (IBM Corp., Armonk, NY, USA). The Kolmogorov-Smirnov test was used to assess the normality of distribution. Continuous variables were expressed as mean ± standard deviation and compared using the Student's t-test or the Mann-Whitney U test, as appropriate, whereas categorical variables were presented as frequencies, and compared using the chi-square test. A two-tailed p-value of less than 0.05 was considered statistically significant.

Results

A total of 1,353 sexually active women were included in the study. Among them, 427 (31.5%) reported anorgasmia, while 926 (68.5%) did not. The mean age of participants was 34.6±7.9 years. Most women were non-employed (77.5%) and had completed only primary school education (60.5%). The proportion of single women was 1.5%.

Anorgasmia was significantly more prevalent among non-employed women [84.1% vs. 71.6%; crude odds ratio =2.09, 95% confidence interval (CI): 1.56-2.82; p=0.0001] compared with employed women, and among those with primary school education (63.7% vs. 58.7%; p=0.001) compared with women with higher education levels. Conversely, single women were more frequently represented in the anorgasmia (–) group, although this subgroup was very small (2.7% vs. none reported; p=0.003). No significant differences were observed between the groups regarding the partner's occupation, number of marriages, or contraceptive methods (p>0.05) (Table 1).

Reproductive and clinical parameters are summarized in Table 2. Age and duration of marriage did not differ significantly between the groups (p=0.712 and p=0.601, respectively). The reported frequency of sexual intercourse per month was similar in both groups (7.31±4.31 vs. 7.13±4.25; p=0.472). However, gravidity and parity were significantly higher in the anorgasmia (+) group compared with the anorgasmia (–) group (3.09±2.04 vs. 2.75±1.70, mean difference =+0.34, 95% CI: +0.12 to +0.56; p=0.001) and (2.15±1.27 vs. 1.96±1.14, mean difference =+0.19, 95% CI: +0.05 to +0.33; p=0.005, respectively). The number of living children was also greater among women with anorgasmia (2.05±1.05 vs. 1.89±1.02; mean difference =+0.16, 95% CI: +0.04 to +0.28; p=0.008). Additionally, vaginal pH was significantly higher in the anorgasmia (+) group (5.27±1.09 vs. 5.14±1.00; mean difference =+0.13, 95% CI: +0.01 to +0.25; p=0.032), indicating a possible association between vaginal environment and orgasmic function.

No significant relationship was observed between HPV positivity and anorgasmia (p>0.05). The presence or genotype of HPV did not appear to influence the occurrence of orgasmic dysfunction. In contrast, sociodemographic factors such as being non-employed and having a low educational level, as well as reproductive factors including high gravidity and parity, showed strong associations with anorgasmia.

Regarding coexisting genital infections, anorgasmia was less prevalent among women with Chlamydia positivity (p=0.05), and no cases of anorgasmia were reported among those diagnosed with Gonorrhea. However, these findings should be interpreted with caution due to the limited number of cases in these subgroups.

Table 1. Sociodemographic characteristics

	Anorgasmia (+) (n=427)	Anorgasmia (–) (n=926)	p-value
Age (years)	34.56±7.88	34.7±8.29	0.712
Non-employed (%)	84.1	71.6	0.0001
Primary school graduate (%)	63.7	58.7	0.001
Single (%)	2.7	-	0.003

Table 2. Reproductive health characteristics

	Anorgasmia (+)	Anorgasmia (–)	p-value
Gravidity	3.09±2.04	2.75±1.70	0.001
Parity	2.15±1.27	1.96±1.14	0.005
Living children	2.05±1.05	1.89±1.02	0.008
Vaginal pH	5.27±1.094	5.141±1.00	0.032

Discussion

This study investigated the relationship between HPV positivity, HPV genotype, and female sexual dysfunction, particularly anorgasmia and psychosexual stress among Turkish women participating in a cervical cancer screening program. The findings revealed that anorgasmia was significantly associated with sociodemographic and reproductive characteristics, such as non-employment, a low educational level, higher gravidity and parity, and elevated vaginal pH, whereas no significant association was observed between HPV positivity or genotype and orgasmic dysfunction. These results suggest that the determinants of female sexual dysfunction in this population are predominantly psychosocial and reproductive rather than virological.

The prevalence of anorgasmia in our cohort (31.5%) is consistent with previous reports indicating that approximately one-third to one-half of women of reproductive age experience some form of orgasmic difficulty[7,8]. Educational attainment, marital relationship quality, and parity are among the strongest predictors of sexual dysfunction across diverse populations[3]. The higher prevalence of anorgasmia among non-employed women and women with lower education levels likely reflects the influence of limited sexual literacy, restricted autonomy, and traditional gender norms prevalent in conservative cultural contexts. Turkish women with lower education levels and higher depressive symptoms have been shown to exhibit greater sexual dysfunction, emphasizing the link between education, mental health, and sexual well-being[9].

Reproductive health parameters including gravidity, parity, and vaginal pH were also identified as significant predictors of anorgasmia. These associations are biologically plausible, as repeated pregnancies and deliveries can result in pelvic floor muscle weakening, hormonal changes, and altered genital vascularization, which collectively diminish orgasmic response and satisfaction. Obstetric factors such as instrumental delivery, episiotomy, and multiparity significantly increase the risk of pelvic floor disorders and related sexual dysfunction later in life[10]. Moreover, the elevated vaginal pH observed among women with anorgasmia may indicate disruption of the normal vaginal microbiome. A Lactobacillus-dominant flora is essential for maintaining mucosal integrity, lubrication, and sexual comfort, and disruption of this environment has been linked to dyspareunia and reduced sexual satisfaction[11,12].

No significant relationship was found between HPV positivity and genotype and anorgasmia. Although some studies suggested that HPV diagnosis could negatively affect sexual function and increase anxiety, these effects appear to be mediated primarily through psychological distress and fear of malignancy rather than through direct biological mechanisms[5,6]. When sociodemographic and reproductive confounders were controlled, HPV status did not independently predict orgasmic dysfunction. This may reflect cultural coping mechanisms, social support, or effective communication between healthcare providers and patients in Türkiye.

The lower prevalence of anorgasmia among women with Chlamydia trachomatis positivity and the absence of cases among those with Neisseria gonorrhoeae infection should be interpreted cautiously, given the small number of infected participants. This pattern could be influenced by behavioral factors such as increased medical attention or heightened sexual health awareness following infection diagnosis.

Overall, these findings reinforce the need to adopt a biopsychosocial perspective when assessing and managing female sexual dysfunction. Women's sexual function is shaped by the dynamic interplay of biological, emotional, and sociocultural elements rather than by physiological factors alone[4,13]. Accordingly, interventions aimed at improving sexual health should integrate medical, psychological, and educational components, particularly in cultures where open discussions of sexuality remain limited.

Study Limitations

This study's strengths include its large sample size, use of validated psychometric tools (FSFI, ASEX, BDI, BAI), and comprehensive evaluation of both biological and psychosocial determinants. However, several limitations should be acknowledged. The cross-sectional design precludes causal inference. Factors such as partner satisfaction, body image perception, and sexual trauma history were not assessed. Moreover, HPV genotypes were not analyzed individually for subtype-specific effects. Future multicenter and longitudinal studies incorporating psychosexual interventions and genotype-specific analyses could further clarify the complex interplay between infection, mental health, and sexual function.

Conclusion

Anorgasmia among Turkish women is primarily influenced by sociodemographic and reproductive variables, particularly being non-employed, lower educational level, high gravidity and parity, and elevated vaginal pH, rather than HPV infection or genotype. These findings highlight the importance of biopsychosocial and culturally sensitive approaches in gynecologic practice, emphasizing sexual education, mental health support, and reproductive counseling to improve overall female sexual health and well-being.

Ethics

Ethics Committee Approval: The study was approved by Antalya City Hospital Ethics Committee and was performed in accordance with the ethical standards described in the 1975 Declaration of Helsinki, as revised in 2000 (approval no: 2025-134, date: 28.05.2025).

Informed Consent: Informed consent was obtained from each patient prior to the study.

Footnotes

Authorship Contributions

Surgical and Medical Practices: C.K., Concept: C.K., Design: C.K., Data Collection or Processing: S.M.G.K., A.P., E.T., M.M.İ., Analysis or Interpretation: S.M.G.K., Literature Search: G.G., E.D., Writing: C.K.

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

Financial Disclosure: The authors declared that this study received no financial support.

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Clinical Investigation / Araştırma

DOI: 10.4274/tjod.galenos.2025.02697

Turk J Obstet Gynecol 2025;22(4):292-8

Comparison of asprosin immunoreactivity in endometrial hyperplasia and grade-1 endometrial adenocarcinoma: A retrospective case-control study

Endometriyal hiperplazi ve grade-1 endometriyal adenokarsinomdaki asprosin immünoreaktivitesinin karşılaştırılması: Retrospektif bir olgu-kontrol çalışması

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Abstract

Objective: It has been demonstrated that asprosin, a glucogenic adipokine released by white adipose tissue, contributes to the pathophysiology of cancer and disorders associated with it. The aim of this study was to compare the immunoreactivity of asprosin in grade I endometrial adenocarcinoma and in endometrial hyperplasia (EH) with and without atypia.

Materials and Methods: A total of 80 cases previously diagnosed with grade 1 endometrial adenocarcinoma and EH with and without atypia, and for which paraffin blocks were obtained, were included in the study. The resulting paraffin blocks were sectioned again and immunostained for asprosin. A total of 80 cases were divided into 4 groups according to their histopathological diagnoses. Group (G) 1 (n=20): proliferative endometrium, G2 (n=20): EH without atypia, G3 (n=20): EH with atypia, G4 (n=20): Grade 1 endometrial adenocarcinoma. Endometrial samples from 80 patients were sectioned, and asprosin immunoreactivity was evaluated by immunohistochemical staining under a light microscope.

Results: In comparison to the proliferative endometrium group, the grade I endometrial adenocarcinoma group had considerably increased asprosin immunoreactivity. However, between the proliferative endometrium group and the groups with endometrial hyperplasia, without atypia, and endometrial hyperplasia, with atypia, there was no significant difference in asprosin immunoreactivity.

Conclusion: While asprosin immunoreactivity scores are higher in grade I endometrial adenocarcinomas, they are similar to those of the proliferative endometrium in cases of EH with and without atypia, suggesting that energy metabolism contributes to the development of cancer arising from endometrial hyperplasia. Asprosin immunoreactivity can be studied as a marker to predict the progression of EH to cancer.

Keywords: Immunohistochemistry, asprosin, endometrial hypeplasia, endometrial cancer

PRECIS: Asprosin may play a role in the pathogenesis of endometrial hyperplasia.

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Received/Geliş Tarihi: 25.08.2025 **Accepted/Kabul Tarihi:** 08.10.2025 **Epub:** 15.10.2025 **Publication Date/Yayınlanma Tarihi:** 02.12.2025

Cite this article as: Şenocak A, Yavuzkır Ş, Atılgan R, Yurt N, Balta H, Hançer S, et al. Comparison of asprosin immunoreactivity in endometrial hyperplasia and grade-1 endometrial adenocarcinoma: a retrospective case-control study. Turk J Obstet Gynecol. 2025;22(4):292-8

Öz

Amaç: Beyaz yağ dokusundan salgılanan glukojenik bir adipokin olan asprosinin, kanser ve ilişkili bozuklukların patofizyolojisine katkıda bulunduğu gösterilmiştir. Bu çalışmanın amacı, atipili ve atipisiz endometriyal hiperplazi ve grade 1 endometriyal adenokarsinomda asprosinin immünoreaktivitesini karşılaştırmaktır.

Gereç ve Yöntemler: Çalışmaya daha önceden grade 1 endometriyal adenokarsinom ile atipisiz ve atipili endometriyal hiperplazi (EH) tanısı almış ve parafin blokları elde edilen toplam 80 olgu dahil edildi. Elde edilen parafin bloklardan tekrar kesitler alınarak asprosin için immünboyama yapıldı. Toplam 80 olgu histopatolojik tanılarına göre 4 gruba ayrıldı. Grup (G) 1 (n=20): proliferatif endometriyum, G2 (n=20): atipisiz EH, G3 (n=20): atipili EH, G4 (n=20): Evre 1 endometriyal adenokarsinom. Seksen hastadan alınan endometriyal örnekler tekrar kesitlendirildi ve asprosinin immünoreaktivitesi ışık mikroskobu altında immünhistokimyasal boyama ile değerlendirildi.

Bulgular: Proliferatif endometriyum grubuyla karşılaştırıldığında, evre I endometriyal adenokarsinom grubunda asprosin immünoreaktivitesi anlamlı olarak artmıştı. Ancak, proliferatif endometriyum grubu ile atipisiz EH ve atipili EH grupları arasında asprosin immünoreaktivitesi açısından anlamlı bir fark yoktu.

Sonuç: Asprosin immünoreaktive skorlarının grade I endometriyal adenokarsinomlarda yüksek olmasına karşın atipisiz ve atipili EH'lerde proliferatif endometriyuma benzer şekilde olması enerji metabolizmasının EH'den kaynaklanan kanser gelişimine katkıda bulunduğunu göstermektedir. Asprosin immünreaktivitesi EH'den kansere dönüşümü tahmin etmede bir belirteç olarak incelenebilir.

Anahtar Kelimeler: İmmünohistokimya, asprosin, endometriyal hipeplazi, endometriyal kanser

Introduction

The abnormal growth of endometrial glands brought on by a relative lack of progesterone and prolonged exposure to estrogen, is known as endometrial hyperplasia (EH)[1]. Histopathological complexity, unusual features, an aberrant gland-to-stroma ratio, and uneven endometrial growth are its defining characteristics. It should be mentioned, though, that untreated cases of EH might result in the development of endometrial cancer (EC)[1-3]. In 2014, the World Health Organization divided EHs into two groups based on whether they had cytological atypia. In this instance, cases with atypia were categorized as endometrial intraepithelial neoplasia, whereas those without atypia were classified as EH[4].

Although the risk of EC is about quadrupled in cases of hyperplasia without atypia, curettage and hormonal therapy are effective in the majority of cases[5]. Since EH is a precursor lesion to EC and has an incidence that is almost three times higher than EC, early diagnosis can prevent the progression to cancer[6]. The transition from hyperplasia without atypia to hyperplasia with atypia and carcinoma is the first stage of endometrial endometrioid cancer. It has been proposed that unopposed estrogen signaling is a key factor in the initiation of EH and its progression to endometrial endometrioid cancer[7]. EC has an overall five-year survival rate of 81% and a 3.1% lifetime risk[8]. Fortunately, because of the early signs of postmenopausal bleeding, the disease is typically limited to the uterus, with a median diagnostic age of 64. Five-year survival rates are 95% when localized disease is found and surgically removed. Five-year survival rates for distant organ disease, however, are only 18%. Medical therapy, radiation therapy, and surgery are the three main methods of treating endometrial cancer[7]. It is projected that in 2023, there will be 13,030 uterine cancer-related fatalities and 66,200 new cases in the United States[9]. These global and national patterns have several underlying causes that are not well understood. Estrogen-related risk factors, including obesity, nulliparity, late menopause, early menarche, and estrogen supplementation during menopause, are linked to almost 80% of endometrial cancers, which are estrogen receptor positive[10].

In certain nations undergoing socioeconomic transition, the rapidly rising incidence of EC may be attributed to changes in fertility and reproductive variables, such as fewer pregnancies and nulliparity. Furthermore, obesity is on the rise globally and is likely a factor in this development. Additional variables to take into account include shifts in the use of perimenopausal hormones, increases in diabetes, declines in smoking incidence, modifications to birth control, and shifts in the rates of hysterectomy[11]. It has been demonstrated that adipose tissue and fat cells contribute to tumor growth and progression[12-14]. White adipose tissue secretes the glucogenic adipokine asprosin, which controls blood sugar levels. The G protein-cAMP-PKA pathway is activated by asprosin, causing the release of glucose into the circulation[15]. Asprosin is mostly found in white adipose tissue, although it is also present in the lung, heart, liver, skeletal muscle, and pancreas[15,16]. Furthermore, it has been demonstrated that asprosin levels are altered in cancer and illnesses that may be linked to cancer[13,17]. Our study's objectives were to investigate asprosin immunoreactivity in patients with grade 1 endometrioid adenocarcinoma, proliferative endometrium, and EH with or without atypia.

Materials and Methods

This retrospective case-control study was approved by the ethical committee and carried out in compliance with the Declaration of Helsinki's principles.

Selection of Cases

Ethical approval was obtained from the Fırat University Non-Interventional Research Ethics Board (date: 13.01.2022, number: 2022/01-07). Endometrium samples (biopsies and resections) obtained between 2010 and 2020 were retrospectively scanned in the archive of the university department of pathology. Once pathology reports were reviewed and previous pathological diagnoses were confirmed, a total of 80 patients were included in the study, with 20 cases in each group.

Group (G) 1 (n=20): Proliferative endometrium
G2 (n=20): EH without atypia
G3 (n=20): EH with atypia
G4 (n=20): Grade-1 endometrioid adenocarcinoma
Blocks from each case were sectioned again and immunohistochemically stained for asprosin.

Immunohistochemistry

Sections with a thickness of 4-6 µm were obtained from paraffin blocks and mounted on polylysine-coated slides. For antigen retrieval, the deparaffinized sections were heated in a citrate buffer solution (pH 6) using a microwave oven (750 W) for 7+5 minutes, following passage through a graded alcohol series. After boiling, the tissues were allowed to cool to room temperature for approximately 20 minutes. Endogenous peroxidase activity was inhibited by washing the tissues with [phosphate buffered saline (PBS), P4417, Sigma-Aldrich, USA] three times for 5 minutes each, followed by incubation in a hydrogen peroxide block solution (Hydrogen Peroxide Block, TA-125-HP, Lab Vision Corporation, USA) for an additional 5 minutes. To minimize background staining, the slides were again washed with PBS (3x5 minutes) and then treated with Ultra V Block solution (TA-125-UB, Lab Vision Corporation, USA) for 5 minutes.

The tissues were subsequently incubated with the primary antibody against asprosin (anti-asprosin antibody, FNab09797, Fine Test, China) diluted 1:200 for 60 minutes at room temperature in a humidified chamber. Following three washes with PBS (5 minutes each), sections were incubated with a secondary antibody (biotinylated Goat Anti-Polyvalent, TP-125-BN, Lab Vision Corporation, USA) for 30 minutes under the same conditions. After another series of PBS washings (3x5 minutes), Streptavidin Peroxidase (TS-125-HR, Lab Vision Corporation, USA) was applied for 30 minutes at room temperature in a humid environment, followed by PBS washings.

For chromogenic visualization, a mixture of AEC Substrate and AEC Chromogen was added until adequate signal development was observed under a light microscope (AEC Substrate, TA-015-HAS, and AEC Chromogen, TA-002-HAC, Lab Vision Corporation, USA). The slides were then rinsed with PBS and distilled water, counterstained with Mayer's hematoxylin, and mounted with the appropriate mounting medium (Large Volume Vision Mount, TA-125-UG, Lab Vision Corporation, USA). Microscopic evaluation and photography were performed using a Leica DM500 microscope equipped with a Leica DFC295 camera.

Immunostaining was semi-quantitatively scored using a histoscore calculated as the product of staining diffuseness and intensity. Diffuseness was graded as 0.1 (<25%), 0.4 (26-50%), 0.6 (51-75%), and 0.9 (76-100%), while staining intensity was rated as 0 (none), +0.5 (very low), +1 (low), +2 (moderate), and +3 (strong). The histoscore was then calculated as (Histoscore = diffuseness x intensity)[18].

Statistical Analysis

Data were presented as mean ± standard deviation. SPSS version 22 was used for statistical analysis. Differences between the groups were analyzed with one-way ANOVA and post-hoc Tukey test. Receiver operating characteristic (ROC) curve analysis was performed to determine the cut-off value of immunoreactivity histoscore values to differentiate between proliferative endometrium and grade I endometrial adenocarcinoma. ROC curve analysis results were presented as % specificity and % sensitivity, with area under the ROC curve (AUC), p-value, and 95% confidence interval (CI). P<0.05 was considered statistically significant in all analyses.

Results

As a result of the evaluation of immunohistochemical staining for asprosin immunoreactivity with light microscopy, the immune reactivity of asprosin was determined as cytoplasmic reactivity. Evaluation of immunohistochemical staining for asprosin immunoreactivity under a light microscope revealed no significant difference in asprosin immunoreactivity between the proliferative endometrium group (Figure 1a) and EH without atypia (Figure 1b, p=0.662); and EH with atypia (Figure 1c, p=0.997).

Asprosin immunoreactivity was significantly increased in Grade I endometrial adenocarcinoma when compared with the proliferative endometrium group (Figure 1d, p<0.001) (Table 1).

In the ROC analysis performed to determine the histoscore values of asprosin immuneactivity for the differentiation of proliferative endometrium and grade I endometrial adenocarcinoma. A cut-off value of >0.6 was found to have 100% specificity and 80% sensitivity (AUC=0.968, p<0.001, 95% CI= 0.857-0.998).

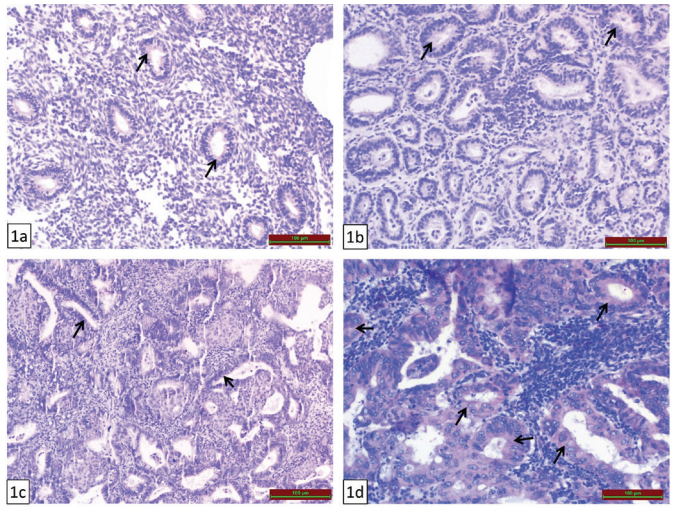

Figure 1. Asprosin immunoreactivity in proliferative endometrium (1a), simple endometrial hyperplasia without atypia (1b), simple endometrial hyperplasia with atypia (1c), complex endometrial hyperplasia without atypia (1d), complex endometrial hyperplasia with atypia (1e), Grade I endometrial adenocarcinoma (black arrow)

Table 1. Asprosin immunoreactivity scores of all groups

Groups	Asprosin immunoreactivity histoscore	p-values
Proliferative endometrium	0.40±0.11	
Endometrial hyperplasia without atypia	0.31±0.12	0.662
Endometrial hyperplasia with atypia	0.36±0.16	0.997
Grade I endometrial adenocarcinoma	0.93±0.35[a]	<0.001

Values are given as mean ± standard deviation.
[a]: Compared with the proliferative endometrium group (p<0.05)

Discussion

The findings of the current study demonstrated that endometrial adenocarcinomas have higher asprosin immunoreactivity than EH and proliferative endometrium without atypia.

During research on neonatal progeroid syndrome, a rare hereditary condition, in 2016, Romere et al.[15]. discovered the protein asprosin to be an adipokine. An increasing number of research since its discovery, indicates that asprosin is useful in controlling metabolic homeostasis and other physiological functions[19]. For instance, it has been demonstrated that asprosin influences hepatic gluconeogenesis and appetite regulation at the hypothalamus level. In addition, there is increasing evidence linking asprosin to intrauterine growth restriction, metabolic diseases, and pregnancy problems, including preeclampsia and gestational diabetes mellitus[20-23]. Studies have shown that long-term high calorie intake causes hypoxia as a result of adipose tissue malfunction leading to oxidative stress and apoptotic pathways[24]. According to Lee et al.[16], asprosin can cause x cells to undergo apoptosis by binding to Toll-like receptor 4 (TLR4) and activating the TLR4/c-JNK-mediated pathway, which raises the levels of proinflammatory cytokines and free oxygen radicals. High levels of oxidative stress and systemic inflammatory pathways are recognized as important in the development of EC as estrogen metabolism[25]. Furthermore, women with polycystic ovary syndrome, which is a significant risk factor for EC along with obesity and diabetes, have been found to have higher levels of circulating asprosin[26]. Studies on the role of asprosin in cancer are limited in the literature. In fact, asprosin therapy of ovarian cancer cells *in vitro* has been demonstrated to change cell communication, transforming growth factor -β signaling, and cell proliferation pathways[27]. It has also been demonstrated more recently that circulating asprosin levels can differentiate between serous benign, serous borderline, and malignant ovarian tumors and may serve as a biomarker in ovarian cancer[28]. In the same vein, there was a notable rise in asprosin immunoreactivity in colorectal adenocarcinoma (i.e., grade 1 versus grade 2), and the clinical value of serum asprosin levels was observed in early pancreatic cancer[29,30]. We demonstrated in our study that asprosin immunoreactivity could be helpful in identifying EC in its early stages. We propose that it could be an especially helpful immunohistochemistry marker for identifying whether EH will eventually progress into cancer.

Protein tyrosine phosphatase receptor type D (PTPRD) is known to regulate several key biological functions, including cell proliferation, differentiation, and neoplastic transformation[31]. A recent genome-wide association study (GWAS) meta-analysis identified a locus within the *PTPRD* gene associated with endometrial cancer. Moreover, emerging evidence indicates that both asprosin and PTPRD may contribute to the regulation of cancer cell growth and metastasis.

Consequently, it manifests as a gynecological malignancy, the fourth most prevalent disease and the third leading cause of cancer-related deaths among women globally[32,33]. Using clinical and pathology samples from both EH and EC cases, we examined asprosin immunoreactivity in these conditions.

Studies have shown that both EC and glioblastoma multiforme (GBM) exhibit significantly reduced PTPRD expression at the gene and protein levels compared with healthy control tissues. According to reports, signaling pathways implicated in cell proliferation may be compromised by this downregulation[34]. For instance, it has been demonstrated that downregulating PTPRD promotes cell proliferation in the RCAS PDGFB/Nestin-tvA glioma mouse model, where the *p16Ink4a* gene is knocked out; whereas restoring PTPRD expression in GBM cells suppresses cell growth and induces apoptosis[35,36]. It has been demonstrated that loss of PTPRD in gastric malignancies causes an increase in CXCL8, stimulating angiogenic and metastatic events through the STAT3 and ERK signaling pathways[37]. Additionally, PTPRD has been implicated in colon cancer cell migration through the β-catenin/TCF/CD44 signaling pathway, and it has been found to function as a tumor suppressor gene in lung cancer[38,39]. In contrast to proliferative endometrium and EH with and without atypia, we demonstrated in our study that asprosin immunoreactivity increased significantly in endometrial cancer. The elevated asprosin immunoreactivity could be attributable to the previously described pathways. This implies that asprosin might be a useful immunohistochemistry marker for identifying risk factors for EC progression. In the same vein, PTPRD functions via the STAT3 pathway, which is triggered in endometrial cancer[40]. In particular, 11.14% of endometrial samples seem to have a mutation in PTPRD[41]. The *PTPRD* gene is associated with one of the 13 loci linked to EC and endometriosis that were found in a GWAS meta-analysis[32]. Even though PTPRD expression was unaffected by the grade or stage of endometrial cancer, it was demonstrated, that obese EC patients had considerably lower levels of PTPRD than healthy weight controls[34]. Notably, it has been demonstrated that the risk of EC increases by 2.0% for those with a body mass index (BMI) of 25-29.9 kg/m^2, 5.2% for those with a BMI of 30 kg/m^2, and 6.9% for those with a BMI of 40 kg/m^2 or

higher[26]. Collectively, these findings suggest that PTPRD plays a key role in EC as well as a potential tumor suppressor gene. PTPRD expression levels in GBM patients may not be clinically useful as a prognostic biomarker. Nonetheless, obesity has been shown to have no effect on PTPRD expression status in these individuals[34].

The effectiveness of some immunohistochemical markers in predicting the probability of transition from EH to EC has been investigated in the literature[42]. Progesterone receptor-B expression[43], COX-2 expression[44], p53 expression[45], lamin receptor-1 expression[46], TRPM2 and TRPM7[18], and hyaluronan synthase 2[47]. immunoreactivities. According to our research, the asprosin immunoreactivity score was similar to cases of proliferative endometrium but far lower than that of endometrial cancer, even in cases of atypical endometrial hyperplasia. This indicates that asprosin might be a useful immunohistochemistry marker worth investigating in the progression from hyperplasia to malignancy.

Metformin has been reported to decrease circulating asprosin concentrations in patients with diabetes mellitus. Gozel and Kilinc[48] demonstrated that plasma and salivary asprosin levels were significantly reduced in newly diagnosed type 2 diabetes mellitus patients receiving metformin therapy. Similarly, Dashtkar et al.[49] observed that metformin treatment alleviated insulin resistance by lowering asprosin levels in both diabetic and control rats. In addition, their study suggested that metformin modulates asprosin and FBN1 expression, indicating possible mechanisms of action extending beyond its effects on insulin sensitivity.

However, asprosin is being investigated as potentially having important roles in receptor dynamics and signaling pathways in EC. It is emphasized that these studies may provide more detailed information about the biological mechanisms by which asprosin influences endothelial cells EC and identify future therapeutic targets. It is reported that asprosin increases cell proliferation and migration through TLR4 or PTPRD signaling, and inhibiting these receptors may offer a new strategy to limit EC progression[50]. However, metformin has been shown to reduce mortality and prolong survival in patients with type 2 diabetes mellitus and EC[51].

Considering the results of existing studies, the use of asprosin inhibitors in addition to progesterone and metformin may be an effective treatment strategy for both reducing the potential for EH to progress to cancer and treating early-stage EC. Further studies on this topic are warranted.

Study Limitations

Limitations of our study include the limited number of cases due to its retrospective nature, the amount of asprosin in tissue and blood could not be measured biochemically. Furthermore, the inability to measure asprosin gene expression is another limitation of our study. Unlike the above studies, the strength of our study is that it asprosin immunoreactivity was compared in cases of proliferative endometrium, EH with and without atypia, and endometrial cancer.

Conclusion

The significant increase in asprosin immunoreactivity in grade I endometrioid adenocarcinoma, compared to EH (with and without atypia), normal proliferative endometrium suggests that molecules related to energy metabolism, in addition to atypia, may play an important role in the transition from hyperplasia to endometrial cancer.

Ethics

Ethics Committee Approval: Ethical approval was obtained from the Fırat University Non-Interventional Research Ethics Board (date: 13.01.2022, number: 2022/01-07).

Informed Consent: Retrospective study.

Footnotes

Authorship Contributions

Surgical and Medical Practices: A.Ş., Ş.Y., R.A., N.Y., Ş.P., B.Ç., Concept: Ş.Y., R.A., T.K., Design: A.Ş., Ş.Y., R.A., Data Collection or Processing: H.B., S.H., T.K., Ş.P., B.Ç., Analysis or Interpretation: A.Ş., Ş.Y., R.A., T.K., Literature Search: A.Ş., Ş.Y., R.A., N.Y., H.B., S.H., Ş.P., M.Y. Writing: Ş.Y., R.A.

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

Financial Disclosure: The authors declared that this study received no financial support.

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Clinical Investigation / Araştırma

DOI: 10.4274/tjod.galenos.2025.10662

Turk J Obstet Gynecol 2025;22(4):299-306

Adjuvant radiotherapy for FIGO 2023 stage IC endometrial carcinoma

FIGO 2023 evre IC endometriyal karsinomda adjuvan radyoterapi

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Abstract

Objective: Within the International Federation of Gynecology and Obstetrics (FIGO) 2023 staging framework, stage IC endometrial carcinoma denotes tumors with aggressive histology confined to the endometrium, without myometrial invasion. This study evaluated treatment outcomes and survival following adjuvant radiotherapy (RT).

Materials and Methods: Twenty-eight patients diagnosed with FIGO 2023 stage IC endometrial carcinoma who were treated with adjuvant RT were retrospectively analyzed.

Results: The most common histologic subtype was serous carcinoma (39%), followed by clear cell carcinoma (25%), high-grade endometrioid carcinoma (25%), carcinosarcoma (7%), and undifferentiated carcinoma (4%). Half of the patients received RT alone, while the remainder received combined RT and chemotherapy. Vaginal brachytherapy was the predominant adjuvant RT technique (86%). The median duration of observation was 59 months. The 2- and 5-year overall survival (OS) rates were 96% and 87; locoregional recurrence-free survival (LRRFS) rates were 96% and 82; and distant metastasis-free survival (DMFS) rates were 92% and 80%, respectively. The presence of malignant peritoneal cytology during surgical staging predicted significantly poorer 5-year OS (93% vs. 33%), LRRFS (86% vs. 33%), and DMFS (90% vs. 0%). Within this limited cohort, the addition of chemotherapy to adjuvant RT did not confer a clear survival advantage. No severe treatment-related toxicities were observed.

Conclusion: While patients with FIGO 2023 stage IC endometrial carcinoma typically achieve favorable outcomes after adjuvant RT, malignant peritoneal cytology remains an adverse prognostic factor. In this subgroup, escalation of adjuvant therapy, such as combination chemotherapy, may be appropriate.

Keywords: Endometrial cancer, FIGO 2023, malignant peritoneal cytology, radiotherapy, stage IC

Öz

Amaç: Uluslararası Jinekoloji ve Obstetrik Federasyonu (FIGO) 2023 evreleme sistemine göre evre IC endometriyal karsinom, miyometriyal invazyon olmaksızın endometriuma sınırlı agresif histolojiye sahip tümörleri tanımlar. Bu çalışma, adjuvan radyoterapi (RT) sonrası tedavi sonuçlarını ve sağkalımı değerlendirmeyi amaçlamaktadır.

Gereç ve Yöntemler: FIGO 2023 evre IC endometriyal karsinom tanısı almış ve adjuvan RT ile tedavi edilmiş 28 hasta retrospektif olarak analiz edildi.

Bulgular: En sık görülen histolojik alt tip seröz karsinomdu (%39), bunu berrak hücreli karsinom (%25), yüksek dereceli endometrioid karsinom (%25), karsinosarkom (%7) ve diferansiye olmayan karsinom (%4) izledi. Hastaların yarısı yalnızca RT alırken, diğer yarısı RT ile kemoterapi kombinasyonu ile

PRECIS: In our analysis of International Federation of Gynecology and Obstetrics 2023 stage IC endometrial carcinoma, malignant peritoneal cytology emerged as a significant adverse prognostic factor, even among patients who received adjuvant radiotherapy.

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Received/Geliş Tarihi: 14.07.2025 **Accepted/Kabul Tarihi:** 27.10.2025 **Publication Date/Yayınlanma Tarihi:** 02.12.2025

Cite this article as: Kahvecioğlu A, Yüce Sarı S, Gültekin M, Yiğit E, Arık Z, Usubütün A, et al. Adjuvant radiotherapy for FIGO 2023 stage IC endometrial carcinoma. Turk J Obstet Gynecol. 2025;22(4):299-306

tedavi edildi. En yaygın RT tekniği vajinal brakiterapiydi (%86). Ortanca izlem süresi 59 aydı. İki ve beş yıllık genel sağkalım (GS) oranları sırasıyla %96 ve %87, lokal bölgesel rekürrenssiz sağkalım (LBRS) oranları %96 ve %82, uzak metastazsız sağkalım (UMS) oranları ise %92 ve %80 olarak bulundu. Cerrahi evreleme sırasında malign peritoneal sitoloji saptanan hastalarda 5 yıllık GS (%93'e karşı %33), LBRS (%86'ya karşı %33) ve UMS (%90'a karşı %0) anlamlı derecede düşüktü. Bu sınırlı kohortta, RT'ye kemoterapi eklenmesi belirgin bir sağkalım avantajı sağlamadı. Ciddi tedaviye bağlı toksisite gözlenmedi.

Sonuç: FIGO 2023 evre IC endometriyal karsinomlu hastalar genellikle adjuvan RT sonrasında iyi prognoz gösterse de, malign peritoneal sitoloji olumsuz bir prognostik faktör olmaya devam etmektedir. Bu alt grupta kemoterapi eklenmesi gibi adjuvan tedavi intensifikasyonu uygun olabilir.

Anahtar Kelimeler: Endometriyal kanser, FIGO 2023, malign peritoneal sitoloji, radyoterapi, evre IC

Introduction

Endometrial carcinoma is the most frequent gynecologic malignancy in developed nations and the second most prevalent in lower-income regions[1]. Over time, treatment has evolved into a highly personalized approach, particularly with the identification of novel molecular markers that are closely related to prognosis[2,3]. These advancements have led to changes in current treatment guidelines by categorizing factors that influence treatment decisions as clinicopathological or molecular features[4,5]. Despite the availability of guideline recommendations supported by high-level evidence for most patients with endometrial carcinoma, the optimal treatment approach for those with aggressive histological subtypes without myometrial invasion (MI) remains unclear.

Endometrial carcinoma confined to a polyp or to the endometrial lining is a rare condition that can pose challenges in deciding on adjuvant treatment[6]. Based on the 2020 European Society of Gynaecological Oncology/European Society for Radiotherapy and Oncology/European Society of Pathology guidelines, non-endometrioid carcinomas without MI are categorized as intermediate risk, and postoperative vaginal brachytherapy (VBT) is typically recommended to reduce the likelihood of vaginal relapse[4]. However, since such cases are infrequent, they have rarely been included in randomized trials, resulting in a limited evidence base compared with that for conventional intermediate-risk endometrioid carcinomas. The 2023 revision of the International Federation of Gynecology and Obstetrics (FIGO) classification redefined these tumors (aggressive histologies such as high-grade endometrioid and non-endometrioid carcinomas without MI) as stage IC disease[7]. This recent change emphasizes the need for updated clinical data reflecting real-world outcomes. Accordingly, this study aimed to investigate the oncologic outcomes and prognostic determinants in patients with FIGO 2023 stage IC endometrial carcinoma who underwent adjuvant radiotherapy (RT), with or without additional chemotherapy.

Materials and Methods

A retrospective cohort analysis was performed using data from 1,297 women with histologically confirmed endometrial carcinoma who received adjuvant RT at our institution between 1994 and 2023. Patients were eligible if they had aggressive histologic subtypes (grade 3 endometrioid or non-endometrioid) limited to the endometrial lining or to a polyp, corresponding to FIGO 2023 stage IC disease[7].

Adjuvant treatment decisions for all patients were made at the weekly gynecologic oncology tumor board, with participation by gynecologic oncologists, medical oncologists, radiation oncologists, pathologists, radiologists, and nuclear medicine specialists, all of whom possess expertise in gynecologic oncology. All patients received an initial treatment consisting of total hysterectomy and bilateral salpingo-oophorectomy with or without lymph node dissection (LND). Peritoneal exploration and pelvic washings were performed according to institutional standards; surgical reports were reviewed to document the extent of exploration. All patients received RT, predominantly with high-dose-rate intracavitary VBT. In cases where LND was not performed, external beam radiotherapy (EBRT) was preferred. Systemic chemotherapy, most frequently as six cycles of carboplatin (AUC5) and paclitaxel (175 mg/m^2), was optionally administered entirely before RT, in a "sandwich" schedule (three cycles before and three cycles after RT), or in a sequential schedule. No concurrent chemoradiation was administered. The decision to administer chemotherapy largely depended on clinical practice at that time. No patients received concurrent chemotherapy.

Post-treatment surveillance included physical and gynecologic examinations every three months during the first two years, then at six-month intervals for the next three years, and annually thereafter. Each visit included assessments by both gynecologic and radiation oncologists, as well as routine laboratory tests. Imaging was performed when recurrence was clinically suspected.

Ethical approval was obtained from the Hacettepe University Health Sciences Research Ethics Board (date: 1305.03.2024, number: 2024/05-25).

Statistical Analysis

Data analyses were conducted using SPSS version 23.0 (IBM, Armonk, NY, USA). Survival outcomes, including overall survival (OS), locoregional recurrence-free survival (LRRFS), and distant metastasis-free survival (DMFS), were estimated with the Kaplan-Meier method, and group comparisons were made using the log-rank test. Locoregional recurrence refers to failures within the vagina, pelvic nodes, or para-aortic nodes, whereas distant metastasis (DM) encompasses hematogenous or peritoneal dissemination. Time-to-event intervals were measured

from the date of surgery to the first event or last follow-up. The prognostic relevance of clinicopathologic variables, such as age, LND status, number of dissected nodes, peritoneal cytology, histologic subtype, tumor location, p53 status, hormone receptor status, and adjuvant treatment type, was evaluated by univariate analysis. Because of the limited sample size and low event count, multivariate Cox regression was not performed; statistical significance was defined as $p<0.05$.

Results

The study population consisted of 28 patients. Table 1 summarizes patient, tumor, and treatment characteristics. The most common histology was serous carcinoma (n=11, 39%), and the most common RT technique was VBT alone (n=24, 86%). Four patients who did not undergo LND received EBRT. While three of these patients received EBRT alone, the fourth patient experienced a rapid local recurrence (LR) extending to the distal vagina during adjuvant chemotherapy and subsequently underwent EBRT followed by VBT. VBT was delivered at a median dose of 27.5 Gy (range, 21-28 Gy) in 3-5 fractions, while EBRT was administered at a median dose of 50.4 Gy (range, 45-50.4 Gy) in 25-28 fractions. Adjuvant chemotherapy was administered to 50% (n=14) of patients. The Median number of chemotherapy cycles was 6 (range, 3-6). Five patients (36%) received the entire chemotherapy course prior to RT, seven (50%) received chemotherapy via a sandwich approach, and two (14%) received chemotherapy after RT.

The median duration of observation was 59 months (range: 4-188 months), with 26 patients (93%) having a follow-up exceeding two years. Throughout the follow-up period, seven patients (25%) experienced disease relapse, and the details of these cases are outlined in Table 2. The rate of LR and DM was 10.7% (n=3) and 21.4% (n=6), respectively. Of the three local recurrences observed, two occurred during follow-up after completion of adjuvant RT, whereas one developed while the patient was still receiving adjuvant chemotherapy and was subsequently treated with RT.

The 2- and 5-year rates for OS, LRRFS, and DMFS were 96% and 87%, 96% and 82%, and 92% and 80%, respectively. Table 3 provides a summary of the univariate analysis outcomes. Survival rates tended to be higher among patients who were under 62 years of age, had endometrioid-type tumors, were hormone receptor positive, or were treated with chemotherapy, although these trends did not reach statistical significance. Malignant peritoneal cytology was the sole factor significantly associated with poorer survival; patients with malignant cytology had markedly worse outcomes than those with benign cytology (Figure 1).

The therapy was well tolerated overall, with no instances of acute treatment-related toxicity of grade 3 or higher reported. For RT-related late toxicity, 6 (21%) patients experienced vaginal stenosis and 10 (36%) experienced vaginal dryness; all events were grade 1-2. Of the 14 patients who received systemic therapy, six (43%) experienced late peripheral neuropathy of grade ≤2, which was the only chemotherapy-related toxicity observed.

Table 1. Summary of patient demographics, tumor features, and treatment details

Characteristic	No (%)
Age (median)	62 years (range, 39-77 years)
Surgery	
TH+BSO	4 (14)
TH+BSO+LND	24 (86)
Extent of LND	
Pelvic	6 (25)
Pelvic and Para-aortic	18 (75)
Number of removed lymph nodes, median	33 (range, 2-86)
Peritoneal cytology	
Positive	3 (11)
Negative	25 (89)
Histology	
Serous carcinoma	11 (39)
Clear cell carcinoma	7 (25)
Endometrioid type carcinoma (grade 3)	7 (25)
Carcinosarcoma	2 (7)

Table 1. Continued

Characteristic	No (%)
Undifferentiated carcinoma	1 (4)
Tumor localization	
Limited to a polyp	17 (61)
Confined to endometrium	11 (39)
LVSI	
Present	0 (0)
Absent	21 (75)
Unknown	7 (25)
p53 staining pattern by immunohistochemistry	
Wild-type	5 (18)
Mutated	16 (57)
Unknown	7 (25)
Adjuvant treatment	
RT	14 (50)
RT+CT	14 (50)
RT technique	
VBT alone	24 (86)
EBRT alone	3 (11)
EBRT+VBT	1 (3)
CT sequence	
Prior to RT	5 (36)
Sandwich approach	7 (50)
After RT	2 (14)

BSO: Bilateral salpingo-oophorectomy, CT: Chemotherapy, EBRT: External beam radiotherapy, LND: Lymph node dissection, LVSI: Lymphovascular space invasion, RT: Radiotherapy, TH: Total histerectomy, VBT: Vaginal brachytherapy

Table 2. Characteristics of 7 patients with recurrence

Patient no.	Age (years)	Histology	Peritoneal cytology	p53	LVSI	Treatment	Recurrence type	Recurrence interval (months)	Salvage treatment	Last status
1	57	CS	(+)	WT	(-)	CT→EBRT+VBT	LR+DM (peritoneum)	14	CT	DoD
2	54	SC	(-)	Mut.	(-)	CT→VBT→CT	LR+DM (peritoneum)	114	CT	DoD
3	62	EC	(-)	N/A	(-)	VBT	LR	56	SBRT	AWD
4	70	SC	(-)	Mut.	(-)	VBT	DM (liver)	51	Surgery+CT	ANED
5	69	SC	(-)	Mut.	N/A	VBT	DM (peritoneum)	37	CT	DoD
6	71	EC	(+)	Mut.	(-)	VBT	DM (liver)	27	CT	AWD
7	70	CCC	(+)	N/A	(-)	VBT	DM (peritoneum)	21	CT	DoD

ANED: Alive with no evidence of disease, AWD: Alive with disease, CCC: Clear cell carcinoma, CS: Carcinosarcoma, CT: Chemotherapy, DoD: Died of disease, DM: Distant metastasis, EBRT: External beam radiotherapy, EC: Endometrioid carcinoma, LR: Local recurrence, LVSI: Lymphovascular space invasion, Mut.: Mutated, N/A: Not available, no.: Number, RT: Radiotherapy, SBRT: Stereotactic body radiotherapy, SC: Serous carcinoma, VBT: Vaginal brachytherapy, WT: Wild type

Table 3. Findings from the univariate analysis

	5y OS (%)	p	5y LRRFS (%)	p	5y DMFS (%)	p
Age (years)						
<62 (n=14)	92	0.91	92	0.65	92	0.38
≥62 (n=14)	84		73		69	
LND						
Yes (n=24)	85	0.58	78	0.56	78	0.33
No (n=4)	100		100		100	
N of removed LNs						
<33 (n=14)	75	0.41	69	0.55	70	0.48
≥33 (n=14)	93		86		86	
Peritoneal cytology						
Positive (n=3)	33	<0.05	33	<0.05	0	<0.05
Negative (n=25)	93		86		90	
Histology						
Endometrioid (n=7)	100	0.67	67	0.65	83	0.95
Non-endometrioid (n=21)	81		84		79	
Tumor localization						
Limited to polyp (n=17)	80	0.70	79	0.75	73	0.89
Confined to endometrium (n=11)	100		75		88	
p53						
Abnormal staining (n=16)	89	0.49	90	0.43	77	0.63
Wild-type staining (n=5)	75		75		75	
Unknown (n=7)	86		73		83	
ER						
Positive (n=12)	100	0.23	100	0.23	90	0.07
Negative (n=6)	80		80		53	
Unknown (n=10)	79		71		81	
PR						
Positive (n=9)	100	0.99	100	0.86	100	0.22
Negative (n=8)	86		86		54	
Unknown (n=11)	79		71		81	
Treatment						
RT (n=14)	81	0.76	67	0.47	66	0.21
RT+CT (n=14)	92		92		92	
EBRT						
Yes (n=4)	75	0.18	83	0.86	83	0.97
No (n=24)	88		81		80	

CT: Chemotherapy, DMFS: Distant metastasis-free survival, EBRT: External beam radiotherapy, ER: Estrogen receptor, LN: Lymph node, LND: Lymph node dissection, LRRFS: Locoregional recurrence-free survival, N: Number, OS: Overall survival, PR: Progesterone receptor, RT: Radiotherapy

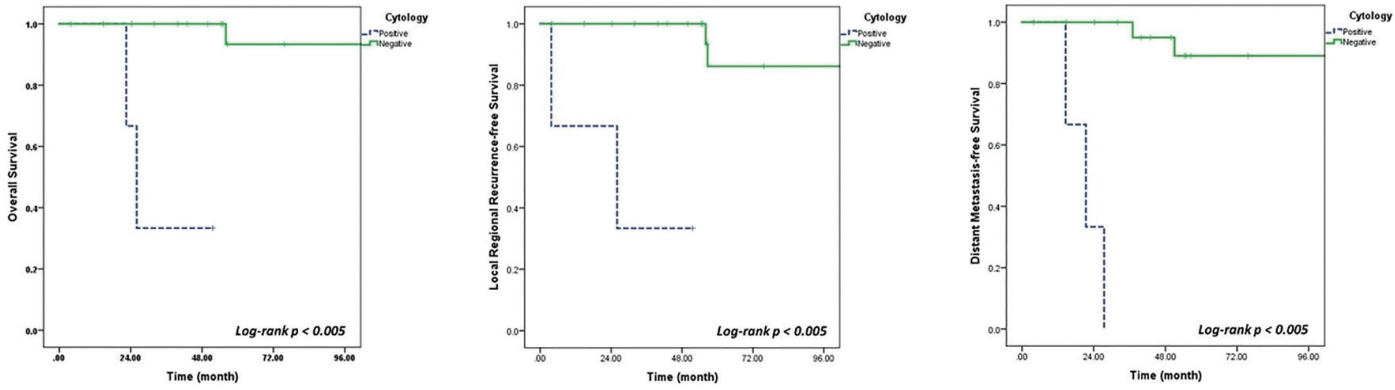

Figure 1. Kaplan-Meier survival curves according to the result of peritoneal cytology

Discussion

This study presents one of the few institutional experiences focusing exclusively on patients with FIGO 2023 stage IC endometrial carcinoma, an uncommon subset characterized by aggressive histology without MI. Our findings demonstrate that adjuvant RT achieves excellent locoregional control with minimal toxicity, while malignant peritoneal cytology remains a strong predictor of poor survival outcomes across all endpoints. Because of the rarity of this entity, prospective data are scarce, and existing evidence largely derives from small retrospective cohorts with heterogeneous designs. Previous series have reported inconsistent outcomes regarding the benefit of chemotherapy or RT in non-myoinvasive, high-grade disease. In the study by Thomas et al.[8], 42 patients diagnosed with stage I endometrial serous carcinoma were evaluated, of whom 15 had tumors without MI. Over a median follow-up of 39 months, none of the patients without MI developed a recurrence, regardless of whether adjuvant treatment was given, and all were alive at 5 years. In contrast, Kelly et al.[9], reported improved survival following adjuvant chemotherapy in a small cohort of 33 patients with serous endometrial carcinoma without MI. Notably, among their Stage I population, no vaginal cuff failures occurred in women treated with VBT; recurrences occurred in approximately one-fifth of those who did not receive it. By comparison, another retrospective study involving 84 women with FIGO 2009 stage I serous or clear cell carcinoma reported that approximately one quarter had tumors confined to the endometrium and that the cohort had a 5-year OS of 84%[10]. In that series, adjuvant chemotherapy did not translate into improved clinical outcomes. A larger population-based investigation encompassing 1,709 women with serous carcinoma confined to the endometrium provided further insight into the role of adjuvant therapy[11]. Roughly half of the patients (51%) underwent postoperative treatment with RT and/or chemotherapy, whereas the remaining 49% were managed by observation alone. That study demonstrated a clear survival advantage for those receiving adjuvant chemotherapy, either alone or in combination with RT, compared with observation. In our cohort, predominant use of a VBT-centered approach resulted in excellent vaginal control, reinforcing its role as an effective modality for local disease management.

Despite inconsistent findings reported across retrospective series, the majority of these analyses focused exclusively on patients with serous or clear-cell histologies, thereby overlooking other aggressive subtypes included in the current FIGO 2023 classification[8,9,11-15]. Although combining distinct histologic entities may not be ideal from a biological standpoint, it should be emphasized that the updated FIGO 2023 classification designates grade-3 endometrioid tumors as aggressive subtypes, aligning them with other high-grade epithelial variants[7]. Hence, more comprehensive studies that collectively evaluate these aggressive histological types are needed. In a recent study by Dallaire Nantel et al.[16], 24% (6 of 25) of patients with grade 3 endometrioid, serous, clear cell, mixed, or carcinosarcoma histology confined to the endometrium or a polyp were treated with VBT, while 76% did not receive adjuvant therapy. In this study, the 3-year progression-free survival and OS rates were very high, being 93% and 100%, respectively. Therefore, researchers indicated that postsurgical follow-up is a safe approach in these cases. In contrast, Chang-Halpenny et al.[17] reported a 5-year OS of 80.6% when 80% of the 46 patients with either serous or clear cell carcinoma confined to the endometrium did not receive any adjuvant treatment. Therefore, other prognostic factors should be considered when deciding whether to administer adjuvant treatment in these cases. Given the results of these studies, one can conclude that adjuvant treatment should be administered to patients with aggressive histologies even if the tumor does not have MI. However, it remains unclear whether this adjuvant treatment should be RT, chemotherapy, or both. Our findings demonstrated satisfactory oncologic outcomes with RT; consequently, vaginal recurrence occurred in only 10.7% of patients. On the other hand, the addition of chemotherapy did not provide a statistically significant survival benefit in this small cohort. However, it should be noted that the 5-year rates of OS, LRRFS, and DMFS in patients that

received chemotherapy were higher than those in patients that received RT alone. This is most likely due to the limited number of individuals and events.

The complexity of determining adjuvant treatment in this uncommon and diverse patient population is further compounded by uncertainty in prognostic factors. As a result, physicians frequently resort to using prognostic factors identified in the literature for broader categories of endometrial cancer patients when addressing this specific subgroup. Although malignant peritoneal cytology is consistently overlooked in staging systems and remains a topic of debate because of conflicting research outcomes, emerging evidence indicates its potential prognostic significance, even among patients with tumors lacking MI[17-21]. Chang-Halpenny et al.[17] demonstrated that malignant peritoneal cytology was associated with an increased risk of recurrence in patients with early-stage serous or clear-cell carcinoma that was limited to or originated from an endometrial polyp. Similarly, in a multi-institutional study involving 33 women with serous endometrial carcinoma confined to a polyp, malignant peritoneal cytology was significantly associated with an increased risk of disease recurrence[18]. In our study, malignant peritoneal cytology emerged as a strong adverse prognostic factor across all survival outcomes. Adjuvant chemotherapy was administered to 33% of patients with malignant cytology; however, likely because of the small sample size, we could not determine the impact of adding chemotherapy on the prognosis of these patients. In light of findings from studies emphasizing the prognostic significance of malignant peritoneal cytology in patients with endometrial cancer, we firmly advocate for its inclusion as a crucial factor when deciding on adjuvant treatment.

Study Limitations

While this study attempts to collectively assess various histologies classified as stage IC according to FIGO 2023, it is crucial to acknowledge its limitations. Firstly, as this is a retrospective analysis including cases treated over nearly three decades (1994-2023), restaging according to the FIGO 2023 system carries inherent limitations. Differences in surgical staging procedures, pathological evaluation, and documentation during this long period may have introduced heterogeneity and a potential risk of understaging. Additionally, the small patient cohort significantly limits the generalizability of our findings. The inherent uncertainty regarding unrecorded factors that might have influenced adjuvant treatment decisions such as comorbidities or institutional preferences, also represents a notable limitation. Moreover, recent advances in molecular classification have reshaped the understanding of endometrial carcinoma biology and prognosis. Findings from The Cancer Genome Atlas indicate that distinct molecular subgroups confer prognostic information that surpasses that provided by traditional histopathologic factors[2,3]. However, molecular stratification was not feasible in our cohort due to the retrospective nature of the study and the lack of available molecular data. Prospective, multicenter investigations incorporating molecular classification are essential to substantiate our observations and refine adjuvant treatment approaches for FIGO 2023 stage IC disease. These factors underscore the need for larger, prospective, and molecularly characterized studies to validate our findings and strengthen evidence-based decision-making in this rare context.

Conclusion

In conclusion, patients diagnosed with FIGO 2023 stage IC endometrial carcinoma who undergo adjuvant RT generally exhibit favorable prognoses. However, our findings underscore the prognostic significance of malignant peritoneal cytology, which remains clinically relevant despite its exclusion from the current staging system. Although overall outcomes are favorable, the presence of malignant peritoneal cytology correlates with poorer survival and may warrant more intensive therapeutic approaches. Future research should focus on evaluating treatment intensification strategies and elucidating the molecular pathways underlying malignant peritoneal cytology. Such efforts will be crucial for optimizing therapeutic decision-making and improving outcomes in this uncommon but clinically important subset of patients.

Ethics

Ethics Committee Approval: Ethical approval was obtained from the Hacettepe University Health Sciences Research Ethics Board (date: 1305.03.2024, number: 2024/05-25).

Informed Consent: Retrospective study.

Footnotes

Authorship Contributions

Surgical and Medical Practices: A.K., S.Y.S., M.G., E.Y., Z.A., A.U., U.A., D.B., N.Ö., F.Y., Concept: A.K., S.Y.S., M.G., E.Y., Z.A., A.U., U.A., D.B., N.Ö., F.Y., Design: A.K., S.Y.S., M.G., E.Y., Z.A., A.U., U.A., D.B., N.Ö., F.Y., Data Collection or Processing: A.K., E.Y., Analysis or Interpretation: A.K., Literature Search: A.K., S.Y.S., M.G., Z.A., A.U., U.A., D.B., N.Ö., F.Y., Writing: A.K., S.Y.S., M.G., F.Y.

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

Financial Disclosure: The authors declared that this study received no financial support.

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Clinical Investigation / Araştırma

DOI: 10.4274/tjod.galenos.2025.21774

Turk J Obstet Gynecol 2025;22(4):307-14

Comparison of survival and apoptosis parameters of ovarian tissue follicles in two vitrification methods of needle-immersion and cryo-support in cancer patients

Kanser hastalarında needle-immersion ve cryo-support olmak üzere iki vitrifikasyon yönteminde over dokusu foliküllerinin sağkalım ve apoptoz parametrelerinin karşılaştırılması

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Abstract

Objective: Ovarian tissue (OT) cryopreservation is a useful technique for preserving fertility potential in women with cancer. Vitrification is a relatively new method. Several devices were discussed. Among these methods, cryo-support and needle-immersion vitrification (NIV) stand out as particularly popular. The aim of this study is the comparison of these devices in terms of follicle quality and DNA status.

Materials and Methods: The OT from 20 cancer patients was transferred with Dulbecco's Phosphate-Buffered Saline supplemented with 5% serum and maintained at 4 °C for 1 hour. After preparation of OT vitrified by two freezing solutions with different concentrations of cryoprotectant, small fragments of OT (~5×1×1 mm) were attached to an insulin needle, and in another group, the tissue fragments (~5×5×1 mm) were loaded onto the Ova Cryo Device Type M, also called cryo-support, and placed in liquid nitrogen. After warming, small tissue pieces were prepared to assess the quality of primordial, primary, and secondary follicles using eosin-hematoxylin staining. An immune-histochemical investigation, including anti-p53 and anti-Caspase 3, was conducted to examine the apoptosis pathway.

Results: The data showed a larger number of high-quality follicles in the cryo-support group ($p<0.05$). Also, the level of apoptosis (p53) molecules is higher in the NIV method ($p<0.05$). However, the levels of caspase 3 were not significantly different between the NIV approach and the cryo-support vitrification group.

Conclusion: A recent study revealed that the cryo-support device is more effective in increasing high-quality follicles and reducing p53, which is associated with early stages of apoptosis. This device may improve clinical outcomes and may be recommended for cryopreservation programs.

Keywords: Ovarian follicle, fertility preservation, vitrification, histological techniques, apoptosis

PRECIS: The study shows cryo-support vitrification better preserves high-quality ovarian follicles and reduces early apoptosis (p53 expression) compared to conventional method, needle-immersion, suggesting improved outcomes for fertility preservation in cancer patients.

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Received/Geliş Tarihi: 04.09.2025 **Accepted/Kabul Tarihi:** 15.10.2025 **Epub:** 17.10.2025 **Publication Date/Yayınlanma Tarihi:** 02.12.2025

Cite this article as: Seify M, Khalili MA, Tafti MH, Dashti S, Khaje Aminian FS, Beigi SAD, et al. Comparison of survival and apoptosis parameters of ovarian tissue follicles in two vitrification methods of needle-immersion and cryo-support in cancer patients. Turk J Obstet Gynecol. 2025;22(4):307-14

Öz

Amaç: Over dokusu (OD) kriyoprezervasyonu, kanserli kadınlarda doğurganlık potansiyelini korumak için yararlı bir tekniktir. Vitrifikasyon, son zamanlarda kullanıma sunulan nispeten yeni bir yöntemdir. Cryo-support ve needle-immersion vitrifikasyon (NIV) yöntemlerinin özellikle popüler olduğu çeşitli cihazlar tartışılmıştır. Bu çalışmanın amacı, bu cihazların folikül kalitesi ve DNA durumu açısından karşılaştırılmasıdır.

Gereç ve Yöntemler: Yirmi kanser hastasından alınan OD, %5 serum eklenmiş Dulbecco Fosfat Tamponlu Salin ile transfer edildi ve 1 saat boyunca 4 °C'de tutuldu. Farklı kriyoprotektan konsantrasyonları içeren iki dondurma solüsyonuyla vitrifiye edilen OD'nin hazırlanmasının ardından, küçük OD parçaları (~5×1×1 mm) bir insülin iğnesine tutturuldu ve diğer bir grupta, doku parçaları (~5×5×1 mm), cryo-support olarak da adlandırılan Ova Cryo Device Type M'ye yüklenerek sıvı nitrojene yerleştirildi. Isıtma işleminden sonra, eozin-hematoksilen boyama kullanılarak primordial, primer ve sekonder foliküllerin kalitesini değerlendirmek için küçük doku parçaları hazırlandı. Apoptoz yolunu incelemek için anti-P53 ve anti-Kaspaz 3'ü içeren bir immünohistokimyasal inceleme gerçekleştirildi.

Bulgular: Veriler, cryo-support grubunda daha fazla sayıda yüksek kaliteli folikül olduğunu gösterdi (p<0,05). Ayrıca, apoptoz (p53) moleküllerinin sayısı NIV yönteminde daha yüksekti (p<0,05). Ancak, kaspaz 3, NIV yaklaşımı ile cryo-support vitrifikasyon grubu arasında anlamlı bir fark göstermedi.

Sonuç: Yakın zamanda yapılan bir çalışma, cryo-support cihazının yüksek kaliteli folikülleri artırmada ve apoptozun erken evreleriyle ilişkili olan p53'ü azaltmada daha etkili olduğunu ortaya koymuştur. Bu cihazın kullanımı klinik sonuçları iyileştirebilir ve kriyoprezervasyon programları için önerilebilir.

Anahtar Kelimeler: Yumurtalık folikülü, doğurganlığın korunması, vitrifikasyon, histolojik teknikler, apoptoz

Introduction

Ovarian tissue cryopreservation (OTC) is a highly effective and promising approach for preserving a considerable number of primordial and primary follicles when the risk of premature ovarian failure is high (>30-50%)[1]. While treatments like chemotherapy, radiation, and bone marrow transplants lead to full recovery for 90% of children with cancer, there's a risk of ovarian failure due to these treatments[2]. In adolescent cancer cases, it is feasible to remove and freeze the ovaries before the depletion of ovarian follicles occurs[3,4]. Immature oocytes possess unique traits that make them ideal for freezing, including their small size (<20 µm), minimal granulosa cells, and inactive metabolism[5,6].

A meta-analysis conducted by Pacheco and Oktay[7] found that using cryopreserved ovarian tissues (OT) for autologous transplants, resulted in a live birth rate of 37.7%, demonstrating a satisfactory level of success in clinical settings. Given the positive outcomes from OTC and ovarian tissue transplant (OTT), the American Society for Reproductive Medicine Committee advocates for this approach as standard practice for young cancer patients aiming to preserve their fertility[8]. Despite these successes, there's a need to enhance follicle viability post-cryopreservation, increase the live birth rate, and extend the lifespan of ovarian auto-grafts, which currently last an average of 27 months per transplant[9].

Several studies have compared vitrification with slow freezing[10,11]. Abir et al.[12], 2017 examined the combination of slow freezing and needle-immersion in terms of proliferation, apoptosis, and follicles in OT, and deemed the slow method a superior technique. In addition, Sugishita et al.[13] investigated cryopreservation by slow freezing. Their results showed that both methods were remarkably similar. According to a recent meta-analysis, vitrification may be more successful than slow freezing in terms of primordial follicular DNA strand breakage and stromal cell preservation[14]. Vitrification is a rapid freezing technique that involves subjecting samples to high concentrations of cryoprotectant solutions and freezing at extremely low temperatures (-130 °C). This method prevents ice crystal formation, which could damage cells during freezing. Several vitrification techniques are available, such as the cryo-support technique, identified by cryo-support (Kitazato Ova Cryo Device Type M, Japan) and the needle-immersion vitrification (NIV) method[15]. The cryo-support device consists of four fine stainless needles and is designed for the cryopreservation of OT. A recent report found no differences in intact primordial follicle survival, DNA damage, and apoptosis rates between cryopreservation support and slow-freezing methods[13].

The purpose of this study was to examine two NIV cryo-support devices in terms of the number of follicles, survival, apoptosis, and DNA breakage. To the best of our knowledge, the above devices have not been examined in detail yet.

Materials and Methods

Samples

A total of 20 cancer patients under the age of 38 years were referred to the Department of Gynecology, Shahid Sadoughi Hospital, Yazd, Iran, and enrolled in this study. The female patients were diagnosed with moderate forms of cervical and uterine malignancies, and were seeking fertility preservation. An ovarian biopsy or OT resection was performed during therapeutic surgery by laparotomy or laparoscopy. These patients underwent OT removal, and the fine ovarian morphology showed no signs of metastasis. Following the completion of the surgical procedure, a portion of the OT was collected and transported to the fertility preservation laboratory with Dulbecco's Phosphate-Buffered Saline (DPBS) supplemented with 5% human serum albumin maintained at 4 °C for 1 hour. The OT underwent three washes in the DPBS medium to ensure the absence of blood. Subsequently, the medulla tissue was excised, and the ovarian cortex was cut into small pieces for freezing. OT were randomly placed in one of the cryo-support or NIV groups (n=10 for each group). This research protocol was reviewed and approved by the Ethics Committee

of the Yazd Reproductive Sciences Institute (approval number: IR.SSU.RSI.REC.1401.008 date: 04.09.2022).

Vitrification Procedure

The Kagawa et al.[16] method was used for vitrification/warming with some modification in tissue loading. Two freezing solutions were used in this method. First, the cortical pieces were submerged in a balancing medium containing Tissue Culture Medium 199 (TCM199) (Life Technologies, CA, USA) supplemented with 20% serum albumin [handling medium (HM)], 7.5% ethylene glycol (EG), and 7.5% dimethyl sulfide (DMSO) for 25 min. The second stage involved freezing the tissue in the vitrification solution (VS), which contains HM with 20% EG, 20% DMSO, and 0.5 M sucrose. Tissue pieces were immersed in VS medium for 15 min. Both stages were done on the shaker at 4 °C. The tissue pieces were separated into two groups.

Needle-immersion Vitrification Method

After freezing, small fragments of OT (~5×1×1 mm, in length, width, and thickness, respectively) were attached to an insulin needle and placed in liquid nitrogen (LN2) with a minimum freezing protection medium[9]. The frozen fragments were transferred into 2.0 mL cryogenic vials (BD Bioscience, San Jose, CA, USA) and stored in the nitrogen tank (Figure 1).

Cryo-support Device

The tissue fragments (~5×5×1 mm) were loaded onto the cryo-support device (Ova Cryo Device TypeM®; Kitazato BioPharma Co., Shizuoka, Japan). Then, they were placed into a cryogenic vial, inserted into the LN2, and subsequently stored in a nitrogen tank (Figure 2).

Thawing of Ovarian Tissue

First, the cryo-vials were removed from the LN2 and placed at room temperature for 1 min. The tissues were transferred into the first medium, containing 40 mL of HM supplemented with 1.0 mol/L sucrose, for 1 min. Then, they were placed in the second medium containing 15 mL HM with 0.5 M sucrose for 5 min and washed three times in the HM for 10 min. After thawing/warming, the ovarian cortical pieces were cut into 1×1 mm pieces for further evaluation to ensure the equal size of tissues in both groups. Small tissue pieces were placed in 100 µL of culture media containing TCM199 (Life Technologies, CA, USA) supplemented with 10% HAS, 100 IU penicillin/mL, and 100 µg streptomycin/mL (Life Technologies). Tissue pieces were incubated in a humidified atmosphere with 5% CO_2 at 37.0 °C for 4 hours. This period enabled the activation of pathways related to DNA damage and programmed cell death. After culture, the studied groups were kept in 10% formalin for 48 hours. These samples were used for hematoxylin-eosin (H&E) staining and immunohistochemistry.

Histological Studies

H&E staining was performed to assess the condition and density of primordial (oocytes surrounded by less differentiated squamous granulosa cells), primary (oocytes surrounded by a single layer of cuboidal granulosa cells), and secondary follicles, which consist of more granulosa cells and contain small accumulations of fluid in the intracellular spaces called follicular fluid. The OT was fixed in 10% buffered formalin (Sigma-Aldrich, Germany) for 2 days. The tissues were then subjected to tissue processing, which included dehydration in increasing concentrations of ethanol and paraffin (Sigma-Aldrich, Germany), followed by embedding. The ovarian cortex was processed by slicing it into 5 µm thick sections. Each slice was stained with H&E (Vetec, São Paulo, Brazil) for light microscopic examination (Olympus, Tokyo, Japan) at 400x magnification. The quality of the follicles was determined by their morphology, which included the nucleus, cytoplasm, and granulosa cells. The follicles were classified into two categories: good quality follicles, with an intact oocyte, well-organized granulosa cells, and no condensed nuclear chromatin; otherwise, they were considered bad quality morphology[17].

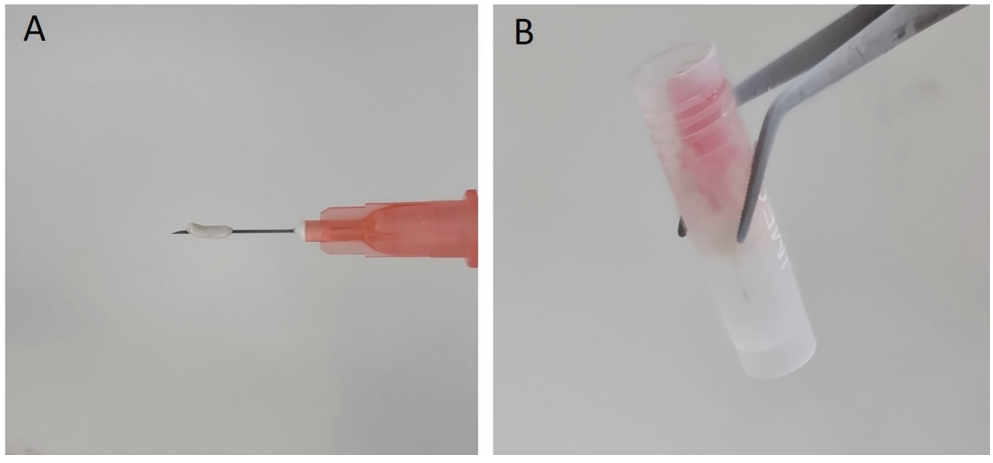

Figure 1. Needle-immersion vitrification method. A: A small fragment of OT (~5×1×1 mm) attached to an insulin needle, and B: It was transferred into 2.0 mL cryogenic vials and stored in the nitrogen tank

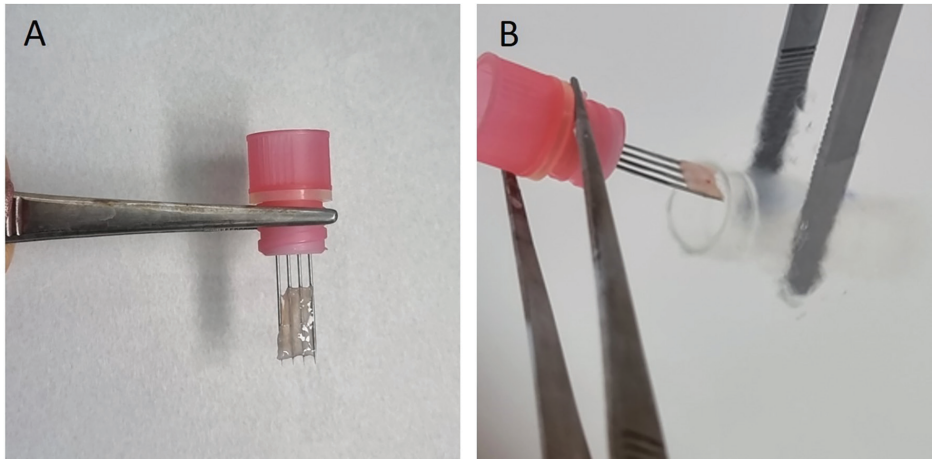

Figure 2. Cryo-support device. A: The tissue fragment (~5×5×1 mm) was loaded onto the cryo-support device, and B: It was inserted into the LN2, placed into a cryogenic vial, and subsequently stored in a nitrogen tank

Immuno-histochemical Dyeing

The initial process and end of apoptosis were investigated using an immunohistochemical approach. The p53 antibody was used to identify the early apoptotic pathway. Antibody Caspase 3 was used to identify the final pathway that leads to DNA damage. Two antibodies were utilized for immune-histochemical investigation, including anti-p53 (Santa Cruz Biotechnology, Sc-126) and anti-Caspase 3 (CPP324-1-18) (Santa Cruz Biotechnology, sc-56052). The slices were incubated for 30 min at 97 °C, then hydrated in a series of alcohol solutions, and finally, they were immersed in 1% hydrogen peroxide in methanol for an additional 30 min. After that, it was washed with Phosphate-Buffered Saline - Tween buffer (PBS-Tween 0.05%), and the antigens were restored with citrate buffer for 30 min at 97 °C. As all of these proteins are cytoplasmic, Triton X-100 was used to penetrate the cell membrane for 10 minutes. To prevent the unexpected response, a 10% goat serum solution was used for 30 min. The slices were then treated with primary antibodies overnight at 4 °C. The tissue slices were washed multiple times. The samples were then treated with secondary antibodies (Master Diagnosis, MAD-000237Q) for 2 h at 37 °C. After washing, DAB solution (3,3' diaminobenzidine) was added to the slices. Then they were stained with hematoxylin, dehydrated using an ascending series of alcohols, clarified in xylene, and mounted for examination at a magnification of 40×.

Statistical Analysis

Normality was analyzed using the Kolmogorov-Smirnov test and then assessed using the Independent samples t-test for parametric data and the Mann-Whitney U test non-parametric data. The data were analyzed using SPSS software version 23. The results were reported as mean ± standard deviation, maximum, and minimum, with a significance threshold of $p<0.05$. All charts were also drafted using the GraphPad Prism V8 application.

Results

There are no significant differences in female parameters in terms of age (p=0.31), anti-Müllerian hormone (p=0.80), follicle stimulating hormone (p=0.58), and obtained fragments of each ovary (p=0.72) between the two groups (Table 1). There was no significant difference in the number of primordial, primary, and secondary, follicle types between the NIV and ova-cryo devices (Table 2). The follicular granulosa cell arrangement and oocyte baseline integrity were better preserved in ova cryo-devices compared to NIV groups (Figure 3). However, compared to the group undergoing NIV, those who underwent cryo-support freezing showed a greater number of high-quality follicles (Table 3).

Our data showed that the quantity of positive apoptosis (p53) molecules was significantly higher in the NIV method than

Table 1. Comparative evaluation of laboratory characteristics of cancer female between NIV and cryo-support groups

Groups Female parameters	NIV group (n=10)	Cry-support group (n=10)	p-value
Age (year)	32.8±5.9	33.7±6.9	0.31*
AMH (ng/mL)	2.08±0.56	2.14±0.47	0.80#
FSH (IU/L)	6.7±0.81	6.5±0.5	0.58#
Mean number of fragments	5.5±2.4	5.1±2.4	0.72#

Data were presented as mean ± standard deviation. *: Statistical analysis was performed based on Mann-Whitney U test, #: Based on independent sample t-test, AMH: Anti-Müllerian hormone, FSH: Follicle stimulating hormone, NIV: Needle-immersion vitrification

in the cryo-support freezing group in terms of primordial (p=0.03), primary (p=0.007), and secondary follicles (p=0.002) in Figure 4.

Immuno-histochemical results revealed that caspase 3 was not significantly different in the freezing groups using the NIV approach, compared to the cryo-support-freezing group in terms of primordial (p=0.13), primary (p=0.22), and secondary follicles (p=0.8) (Figure 5).

Discussion

This study aimed to investigate two methods of OTC: NIV and cryo-support, which are conventional procedures in the vitrification process. The results demonstrated that a cryo-support device could greatly enhance the quality and number of surviving follicles. In this procedure, the expression of the apoptosis-promoting molecule (p53) in follicles was significantly decreased compared to the immersion method with a needle, in cryo-support. This suggested a potential role of cryo-support in minimizing apoptosis during the cryopreservation process.

Currently, there are two common procedures for OT freezing: slow freezing and vitrification. Studies have shown over 150 recorded live births through OTC until 2021[18]. The five largest European centers reported a pregnancy rate of 29% and a live birth rate of 23%[7,19].

The vitrification method is a relatively new approach in OTC, and only a few live births have been documented so far.

Table 2. Comparison of the number of primordial, primary, and secondary follicles in two freezing groups using the NIV and cryo-support groups

Groups	Number of primordial means ± SD (min-max)	Number of primary means ± SD (min-max)	Number of secondary means ± SD (min-max)
Cryo-support	**27.30±6.63 (15-35)**	**19.50±5.93 (13-27)**	7.6±2.31 (6-11)
Needle-immersed	**27.20±5.84 (19-35)**	**19.90±4.22 (14-26)**	7.8±2.04 (5-11)
p-value	**0.97**	**0.86**	0.84

Data were presented as mean ± SD. Statistical analysis was performed based on an Independent sample t-test. P-value was considered <0.05, NIV: Needle-immersion vitrification, SD: Standard deviation, min: Minimum, max: Maximum

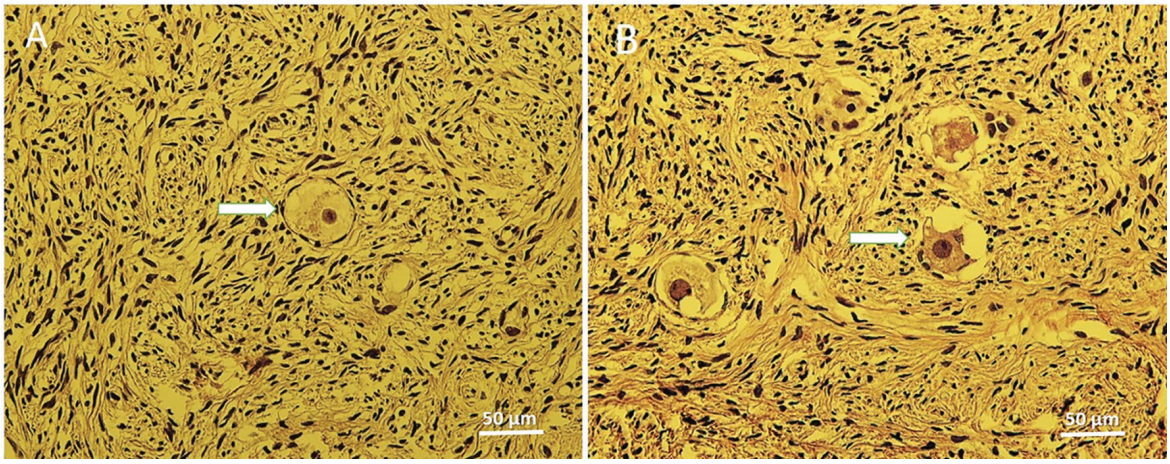

Figure 3. The quality of follicles after vitrification/warming in both groups. A: A primordial follicle with an intact oocyte and well-organized granulosa cells and no condensed nuclear chromatin were categorized as good quality morphology, and B: Primordial and Primary follicles were considered as bad quality morphology. The arrows show primordial follicles

Table 3. Comparison of the number of good quality follicles in two freezing groups NIV and cryo-support groups

Groups	Good quality primordial means ± SD (min-max)	Good quality primary means ± SD (min-max)	Good quality secondary means ± SD (min-max)
Cryo-support	16.60±4.40 (10-23)	6.70±2.31 (4-11)	4.5±1.26 (3-7)
Needle-immersed	12.80±2.82 (9-19)	4.30±1.70 (2-7)	3.3±0.94 (2-5)
p-value	**0.034#**	**0.017#**	0.028*

Data were presented as mean ± SD. *: Statistical analysis was performed based on Mann-Whitney U test, #: Based on Independent sample t-test. P-value considered <0.05, NIV: Needle-immersion vitrification, SD: Standard deviation, min: Minimum, max: Maximum

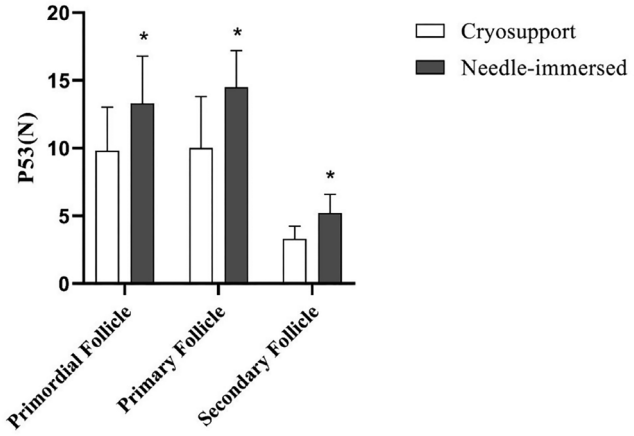

Figure 4. Comparison of the number of primordial, primary, and secondary follicles containing the apoptosis (p53) progressive molecule in two freezing groups, needle-immersion vitrification and cryo-support. All values are presented as the mean ± standard error of the mean. P-value is considered <0.05. Primordial (p=0.03), primary (p=0.007), and secondary follicles (p=0.002)

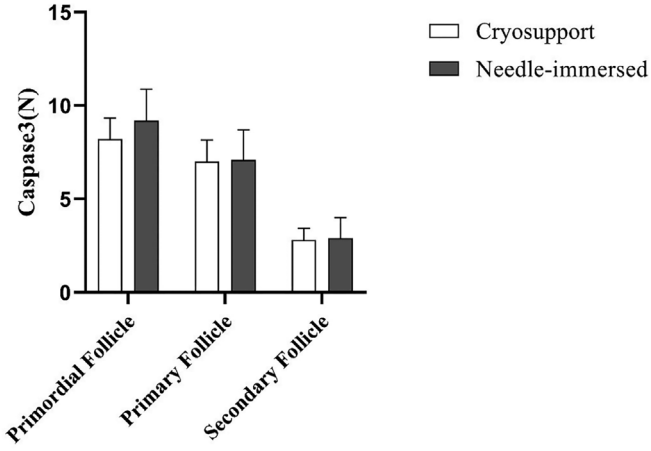

Figure 5. Comparison of the number of primordial, primary, and secondary follicles containing the leading molecule caspase 3 in two freezing groups, needle-immersion vitrification and cryo-support. All values are presented as the mean ± standard error of the mean. P-value is considered <0.05. There was no significant difference between the two groups

The benefits of vitrification include its brief duration during the freezing process and the absence of specific equipment requirements. New research comparing the two methods has shown that there is no substantial difference in clinical outcomes between vitrification and slow freezing methods[7,8].

In a meta-analysis study, Zhou et al.[20] evaluated the number of surviving primordial follicles after vitrification and slow freezing. They concluded that no significant differences were observed in the number of follicles in the freezing groups.

Comparisons of vitrification and slow freezing groups revealed a significant increase in apoptosis levels in both freezing groups, compared to the control. However, there were no significant differences between the freezing groups[21]. In 2015, it was demonstrated that vitrification is a superior method for preserving follicle survival compared to slow freezing. The study found that follicle survival improved to 83.6% in the vitrification group compared to 80.7% in the slow freezing group. Furthermore, there was no significant difference in the amount of DNA breakage between the two groups[22].

In general, a vitrification technique can be classified into open and closed methods[8,23]. An NIV method is a type of open method in OTC. In this method, the OT is placed on a special needle and then immersed in LN2. This method ensures the survival of both follicular and stromal cells[11,15].

This method is simple and requires only a small needle, such as an insulin needle. However, the small size of the OT may create challenges during transplantation. Moreover, a lack of tissue support during direct LN2 exposure can compromise tissue integrity. Cryo-support, another open vitrification method, addresses this issue by offering a stable platform and broader surface area for freezing. It results in better conditions for OTT and improved angiogenesis post-transplantation. By reducing ischemia-related follicle loss, cryo-support may enhance graft viability and functionality[24].

The primordial follicle count is a significant biomarker used to evaluate a woman's ovarian reserve or fertility potential. Since primordial follicles are more resistant to cold damage during the freezing and warming process, their morphology and integrity are often used to assess the quality of OT post-freezing[25]. In our study, we assessed the post-freezing quality of OT and found that the cryo-support method better preserved primordial follicle structure and overall cortical tissue integrity compared to NIV. Additionally, a significant reduction in p53 expression further supported reduced apoptosis in the cryo-support group.

It's worth noting that various factors—including freezing method, cryopreservation media composition, cryoprotective agents, processing time, temperature, and freezing devices—can all affect the quality of OT follicles during freezing and thawing[26-28].

Reducing the thickness of the OT cortex minimizes ice crystal formation. Additionally, thinner fragments allow for deeper penetration of cryoprotectants, leading to proper hydration and cryoprotection[29]. Since the tissue is positioned on stainless steel, cryo-support ensures uniform and rapid exposure to ultra-low temperatures when immersed in LN2, promoting optimal vitrification.

In 2021, Sugishita et al.[13] compared vitrification and slow freezing for preserving primordial and primary follicles. They reported significant follicle loss in freezing groups, though survival and DNA strand breakage rates were not significantly different between open and closed vitrification methods.

Also, we observed that the cryo-support group had a lower expression level of p53 compared to the NIV group. This process,

which involves complete tissue trimming and mounting on cryo-support needles, facilitates even and controlled freezing in LN2. The effects of vitrification on the quantity of apoptosis in ovarian follicles are ambiguous. Although the vitrification method and kind of device are typical for freezing oocytes and embryos, their efficacy for freezing OT has been questioned, and the results are controversial[30].

Recently, Gupta et al.[31] assessed the influence of several vitrification techniques on apoptosis and growth-related gene expression. Their study showed that the percentage of viable pre-antral follicles obtained after vitrification was significantly lower than before freezing. In addition, the expression of the apoptotic gene BAX was recorded as higher in vitrification interventions with different protocols, and the expression of other apoptosis-related genes, such as BCL2 and Caspase 3, did not show any significant differences between the groups. Their results confirm that freezing induces cellular apoptosis. The presence of intracellular caspase is a clear marker of apoptosis, because caspases, particularly caspase 3, are exclusive proteases of apoptosis.

Kometas et al.[32] concluded that there was no significant difference in the quantity or form of OT follicles between vitrification and slow freezing. Vitrification protocols involve using high concentrations of permeable cryoprotectant like EG and DMSO as well as impenetrable cryoprotectant like sucrose, to facilitate cellular dehydration and prevent the formation of ice crystals inside and outside the cell.

A meta-analysis conducted by Shi reviewed 14 studies to evaluate the efficacy of tissue vitrification, despite several research findings showing a decrease in the percentage of apoptosis with vitrification. The study revealed that the effects of freezing were influenced by various parameters and component size, and that vitrification caused significantly less DNA follicular damage compared to slow freezing. Additionally, it was observed that vitrification had a positive impact on the ovary's preservation and the rate of cooling. It may be more successful than slow freezing in terms of preserving stromal cells and OT[14].

The Sugishita et al.'s[13] study utilized γH2AX to evaluate DNA damage and AC3 antibodies to analyze caspase 3 levels, comparing the levels of apoptosis in primordial and primary follicles among different groups, including the cryo-support group. The study concluded that there were no significant differences in the rates of DNA damage between the cryo-support and the other groups.

Study Limitations

One limitation of this study was the small sample size due to the challenge of obtaining healthy OT for comparison from cancer patients. Nonetheless, the consistent improvements in follicle quality observed with cryo-support emphasize its potential clinical value.

Conclusion

Currently, no studies directly compare NIV and cryo-support methods for vitrification. Our study fills this gap and highlights the benefits of cryo-support in preserving follicular integrity. A recent investigation revealed that the cryo-support device preserved higher-quality follicles and reduced p53 expression, indicating a decrease in apoptosis. These findings suggest that cryo-support may be a promising tool for optimizing OTC and improving clinical outcomes.

Ethics

Ethics Committee Approval: This research protocol was reviewed and approved by the Ethics Committee of the Yazd Reproductive Sciences Institute (approval number: IR.SSU.RSI.REC.1401.008 date: 04.09.2022).

Informed Consent: Retrospective study.

Footnotes

Authorship Contributions

Surgical and Medical Practices: M.H.T., F.S.K.A., S.A.D.B., **Concept:** M.S., M.A.K., S.D., F.A., Design: M.S., M.A.K., S.D., F.A., Data Collection or Processing: M.S., M.H.T., F.S.K.A., S.A.D.B., F.A., Analysis or Interpretation: M.S., S.A.D.B., F.A., Literature Search: M.S., F.A., Writing: M.S., M.A.K., M.H.T., S.D., F.S.K.A., S.A.D.B., F.A.

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

Financial Disclosure: The authors declared that this study received no financial support.

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Clinical Investigation / Araştırma

DOI: 10.4274/tjod.galenos.2025.05082

Turk J Obstet Gynecol 2025;22(4):315-20

Secretome improves anti-Müllerian hormone level and ovarian function in a premature ovarian insufficiency mice model

Sekretom, prematüre yumurtalık yetmezliği fare modelinde anti-Müllerian hormon düzeyini ve yumurtalık fonksiyonunu iyileştiriyor

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Abstract

Objective: To evaluate the efficacy of secretome in improving the anti-Müllerian hormone (AMH) level and ovarian weight and restoring ovarian function in the premature ovarian insufficiency (POI) model mice.

Materials and Methods: A randomized, post-test-only control-group design was conducted on 18 mice, which were divided into three groups: A control group and two case groups injected with a secretome. Blood samples were analyzed for the AMH level with an enzyme-linked immunosorbent assay kit; ovarian weight was measured; and hematoxylin-eosin staining was used to measure and categorize follicles at each stage.

Results: The cyclophosphamide (CTX) group showed significant differences in ovarian weight, AMH, and the numbers of primary, secondary, antral, and atretic follicles compared with the control group, indicating induction of premature ovarian failure. Follicular development was improved in the CTX-secretome group compared to the CTX group, with significantly increased ovarian weight and AMH, increased numbers of primary, secondary, and antral follicles, and decreased numbers of atretic follicles. However, the results showed a significant difference between the CTX-secretome and the control group.

Conclusion: Our findings show that secretome therapy improved POI management, but the results have not yet restored normal ovarian function. It still does not achieve the same functional state as normal ovarian function. Further research, particularly involving different doses of secretome, is necessary to validate these findings.

Keywords: Primary ovarian insufficiency, anti-Müllerian hormone, secretome, ovarian function tests

Öz

Amaç: Prematüre over yetmezliği (POY) model farelerde anti-Müllerian hormon (AMH) düzeyini ve over ağırlığını iyileştirmede ve over fonksiyonunu geri kazandırmada sekretomenin etkinliğini değerlendirmektir.

Gereç ve Yöntemler: On sekiz fare üzerinde, yalnızca test sonrası kontrol grubu tasarımı uygulanarak, fareler üç gruba ayrıldı: Bir kontrol grubu ve sekretom enjekte edilmiş iki olgu grubu. Kan örnekleri, enzim bağlantılı immünosorbent test kiti ile AMH düzeyi açısından analiz edildi; yumurtalık ağırlığı ölçüldü; ve her aşamada folikülleri ölçmek ve kategorize etmek için hematoksilen-eozin boyama yöntemi kullanıldı.

PRECIS: We have evaluated that secretome therapy improved premature ovarian insufficiency management in mice model experimental design.

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Received/Geliş Tarihi: 09.09.2025 **Accepted/Kabul Tarihi:** 23.11.2025 **Publication Date/Yayınlanma Tarihi:** 02.12.2025

Cite this article as: Kawilarang S, Wiratnaya IGE, Yasa IWPS, Budiana ING. Secretome improves anti-Müllerian hormone level and ovarian function in a premature ovarian insufficiency mice model. Turk J Obstet Gynecol. 2025;22(4):315-20

Bulgular: Siklofosfamid (CTX) grubu, kontrol grubuyla karşılaştırıldığında over ağırlığı, AMH ve primer, sekonder, antral ve atretik folikül sayıları açısından anlamlı farklılıklar gösterdi ve bu da erken over yetmezliğinin indüklendiğini gösterdi. CTX-sekretom grubunda foliküler gelişim, CTX grubuna kıyasla iyileşti; over ağırlığı ve AMH anlamlı şekilde arttı, primer, sekonder ve antral folikül sayıları arttı ve atretik folikül sayıları azaldı. Ancak sonuçlar, CTX-sekretom grubu ile kontrol grubu arasında anlamlı bir fark olduğunu gösterdi.

Sonuç: Bulgularımız, sekretom tedavisinin POY yönetimini iyileştirdiğini, ancak sonuçların henüz normal yumurtalık fonksiyonunu geri kazandırmadığını göstermektedir. Yine de normal yumurtalık fonksiyonuyla aynı işlevsel duruma ulaşmamaktadır. Bu bulguları doğrulamak için, özellikle farklı sekretom dozlarını içeren daha fazla araştırmaya ihtiyaç vardır.

Anahtar Kelimeler: Primer over yetmezliği, anti-Müllerian hormon, sekretoma, over fonksiyon testleri

Introduction

Premature ovarian insufficiency (POI) is defined as the cessation of ovarian function before the age of 40[1]. The incidence is approximately 1% in women under 40 years of age and 0.1% in women under 30 years of age[2]. Due to the side effects of menopause at an earlier age, women are advised to take hormonal replacement therapy (HRT) until the natural age of menopause, which is around 51 years[3]. However, some contraindications and side effects related to HRT may occur. One of the most challenging issues is whether this occurs in women who want to preserve their fertility or are trying to conceive.

The incidence of cancer has been increasing recently, especially in younger age groups, with approximately 10% of the 6.6 million young adults aged 15-39 years diagnosed with cancer annually[4]. One current approach to cancer management is the use of chemoradiotherapy to improve survival. However, chemoradiotherapy can alter DNA synthesis and the RNA transcriptome, and indirectly accelerate cell death, particularly by reducing the number of dormant follicles in the ovary[5,6]. This process is recognized as an emerging cause of POI in women younger than the typical age at menopause.

Some studies on stem cells have emerged that aim to improve ovarian function. Because of limitations of stem cells, such as the risk of rejection and high cost, the application of the secretome for certain diseases is emerging. The secretome, which consists of proteins, including extracellular matrix proteins, vesicle proteins, and proteins shed from the cell membrane, has the potential to promote angiogenesis, reduce inflammation, and evade immune responses, which together may facilitate restoration of ovarian function and fertility[7].

Anti-Müllerian hormone (AMH) is recognized as a biomarker for assessing functional ovarian reserve[8]. In the ovary, AMH is expressed by granulosa cells of preantral and early antral follicles, indicating the presence of an active follicular pool. A decline in AMH levels reflects a reduction in the number of primordial and developing follicles. Unlike other hormonal markers, AMH is minimally affected by menstrual cycle fluctuations, making it a reliable parameter for evaluating ovarian reserve[8-10].

This research aims to evaluate ovarian function by assessing ovarian reserve through AMH serum levels measured in peripheral blood and by counting follicles on hematoxylin-eosin (H&E)-stained histological sections after administration of the secretome in a cyclophosphamide (CTX)-induced menopausal mouse model.

Materials and Methods

Study Design

The institution at Universitas Udayana approved this experimental study. The Research Ethics Committee, Faculty of Medicine, Universitas Udayana (approval number: 2713/UN14.2.2.VII.14/LT/2024, date: 11.11.2024). This research was conducted from December 2024 to May 2025 at the Faculty of Veterinary, Universitas Udayana, and at the Histology Laboratory of the Faculty of Medicine, Universitas Udayana Denpasar, Bali.

This study used a posttest-only experimental group design and employed wild-type (n=18) female mice aged 6 weeks (20-22 g), which were housed on a 12-hour light/12-hour dark cycle with free access to mouse food and water. Animal procedures and treatments were conducted at the Veterinary Lab of Universitas Udayana in strict accordance with provisions for the protection of experimental animals.

The sample size was determined based on previous studies using a similar CTX-induced POI model[11], in which six animals per group were sufficient to detect significant hormonal and histological changes with statistical power >0.8 and α=0.05. No formal a priori power analysis was conducted. However, the chosen group size aligns with standard practice for reproducible outcomes in rodent POI models.

The secretome used in this study was generated by Regenic KALBE Laboratory Indonesia [certified under current Good Manufacturing Practices (cGMP)] and manufactured by PT Bifarma Adiluhung, Jakarta, Indonesia (batch number RUCM-SFP-080125-1). The product, known as Regenic Hypoxia UCMSC-Secretome, was derived from human umbilical cord mesenchymal stem cells cultured under hypoxic and serum-free conditions to stimulate secretion of paracrine factors. The conditioned medium was collected, centrifuged to remove cell debris, filtered through a 0.22 µm membrane, and concentrated under cGMP standards. Each vial contained 1.5 mL of sterile secretome, confirmed to be free of cells, mycoplasma, and endotoxin (<0.25 EU/mL).

The protein profile consisted primarily of pro-collagen I (889,550 pg/mL), keratinocyte growth factor (111.93 pg/mL), vascular endothelial growth factor (25.5 pg/mL), basic fibroblast

growth factor (20.69 pg/mL), and stromal cell-derived factor-1 (823.5 pg/mL).

After a two-week acclimatization period, the rats were administered CTX for two weeks. One week after the final CTX dose (day 21), the animals received secretome by intramuscular injection (0.1 mL per injection) every two days (days 22, 24, 26, and 28). Samples for data analysis were collected on day 29.

Premature Ovarian Insufficiency Mice Model Preparation

The 6 weeks old Wistar mice (n=18) were observed for the first 1 week to ensure that all mouse was in their estrous cycle, before they were divided into three groups of 6 animals as follow: Group A: Control group; Group B: CTX group (CTX injection) as the POI group, and Group C: POI with secretome group (CTX followed by secretome injection). All injections were administered intraperitoneally, and the chemotherapy dose was given continuously for 14 days. The control group received 0.2 mL of physiological saline daily, compared with the other two groups. Mice in the POI group were given 50 mg/kg CTX on the first day, then received 4 mg/kg per day from day 2 through day 14 to reliably induce a model of POI while minimizing sample loss. This regimen was selected based on a study by Elahi et al.[11], which showed that a high loading dose followed by lower daily maintenance doses of CTX effectively induces follicle loss, endocrine disruption, and impaired ovarian reserve in rodents, without unacceptable mortality.

Morphological Analysis of Ovarian Follicles

At the end of the experiment, the mice were euthanized. The ovaries were preserved in 4% paraformaldehyde and embedded in paraffin, serially sectioned at 5-6 µm, mounted on glass slides, and stained with H&E for morphological analysis by light microscopy. Follicles with a visible nucleus were recorded to avoid counting the same follicle more than once, and follicle classification was based on published criteria.

Follicles were classified by histological pattern into primary follicles characterized by a single layer of cuboidal cells. secondary follicles with layers of cuboid cells with no visible antrum; antral follicles had layers of cuboidal cells, a fluid-filled space, and a cumulus of granulosa cells. Atretic follicles were defined as follicles with zona pellucida remnants[12,13].

Enzyme-linked Immunosorbent Assay (ELISA) of AMH

The AMH was quantified using the Rat AMH ELISA kit (cat. no. EA0083Ra) from Bioassay Technology Laboratory. Following the experiment, peripheral blood samples were obtained, allowed to clot at room temperature for 10-20 minutes, centrifuged at 2,000-3,000 rpm for 20 minutes, and the supernatant was collected free of sediment. After dilution, add the sample to the sample well and 50 µL of biotinylated antigen to each well. Incubate for 60 minutes at 37 °C. Add 50 µL of avidin-HRP to the standard and sample wells; then add 50 µL of substrate solution A and 50 µL of substrate solution B to each well. Lastly, 50 µL of stop solution was added to each well, and the optical density values were measured at 450 nm using a microplate reader within 10 minutes of adding the stop solution[14].

Statistical Analysis

Each experiment in this research was conducted separately. The findings are presented as mean ± standard deviation and were analyzed using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). The normality of the data was assessed using the Shapiro-Wilk test, and data with a normal distribution were analyzed using one-way ANOVA followed by a post-hoc test. In contrast, the Kruskal-Wallis test followed by the Mann-Whitney test were employed to analyze data that were not normally distributed. Statistical significance was defined as $p<0.05$.

Results

Ovarian Weight

The mean ovarian weight was 0.56±0.01 mg in the control group, 0.31±0.04 mg in the CTX model group, and 0.41±0.01 mg in the CTX with secretome group (Figure 1). The post hoc comparisons among all groups showed that mean ovarian weight in the CTX group was significantly decreased compared with the control group ($p<0.05$), whereas mean ovarian weight in the CTX-secretome group was significantly increased compared with the CTX group ($p<0.05$). Despite this, a significant difference was observed between the CTX-secretome and control groups ($p<0.05$).

Ovarian Follicles

The follicles analyzed in this study were primary, secondary, antral, and atretic (Figure 2).

Compared with the CTX group, the CTX-secretome group showed significant increases in the numbers of primary follicles (147.50±12.94 vs. 56.83±2.92, $p<0.05$; b), secondary follicles (57.00±2.28 vs. 17.33±2.58, $p<0.05$; b), and antral follicles

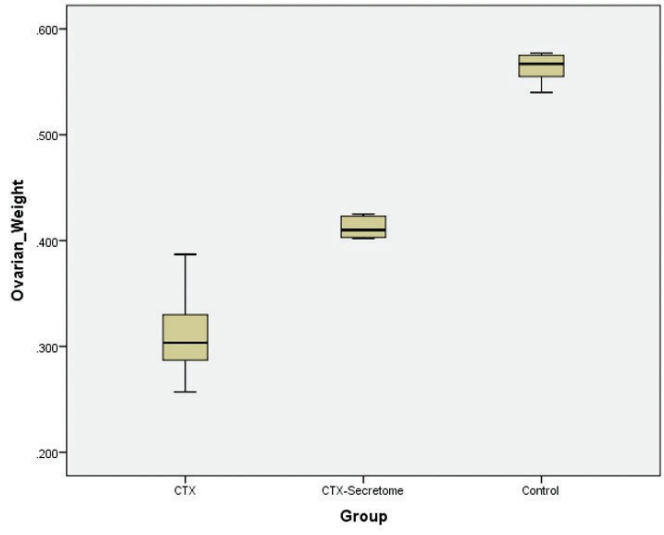

Figure 1. Boxplot graph of ovarian weight

CTX: Cyclophosphamide

(25.33±3.44 vs. 7.50±2.25, p<0.05; b). Compared with the control group, the CTX group demonstrated a significant reduction in all follicle stages: primary follicles (175.33±14.20 vs. 56.83±2.92), secondary follicles (81.50±6.12 vs. 17.33±2.58), and antral follicles (34.50±3.39 vs. 7.50±2.25) (p<0.05; a) (Figure 3).

Meanwhile, the CTX-secretome group showed partial restoration, but remained significantly lower than those of the control group for primary (147.50±12.94 vs. 175.33±14.20), secondary (57.00±2.28 vs. 81.50±6.12), and antral follicles (25.33±3.44 vs. 34.50±3.39) (p<0.05; c).

For atretic follicles, the CTX group showed the highest count (559.17±14.63), which was significantly higher than the counts in the control (354.17±31.53) and CTX-secretome (374.17±12.41) groups (p<0.05; a, b). Secretome treatment reduced the number of atretic follicles to levels not significantly different from the control group (p=0.19; Figure 3c).

Anti-Müllerian Hormone

The AMH, which roughly reflects the number of active follicles in the ovary, also shows improvement following secretome treatment. The AMH was significantly higher in the control group than in the CTX group (11.78±3.51 vs. 5.92±0.74; p<0.05), and was also significantly higher in the CTX-secretome group

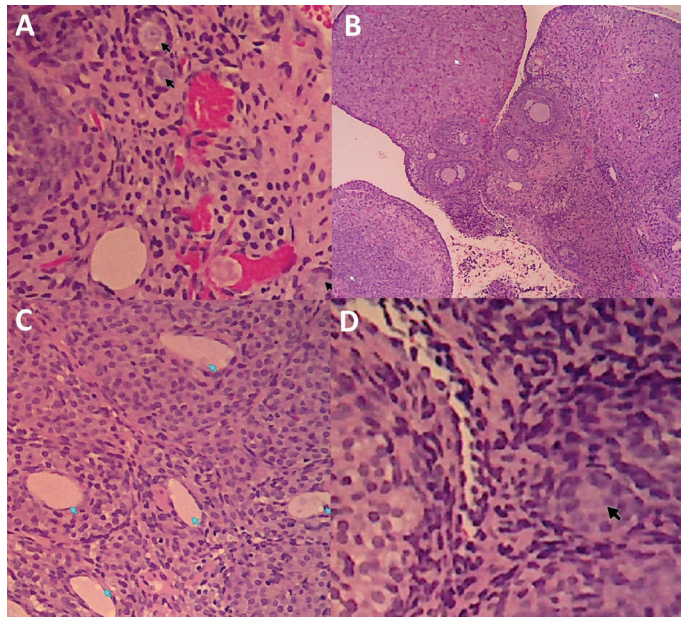

Figure 2. The histology of mouse ovary in the CTX-secretome group with magnification 100x, (A) the distribution of primary follicles (black arrows); (B) the distribution of secondary follicles (purple arrows); (C) the predominance of atretic follicles (blue arrows); (D) the minimal amount of primary follicle (black arrows)

CTX: Cyclophosphamide

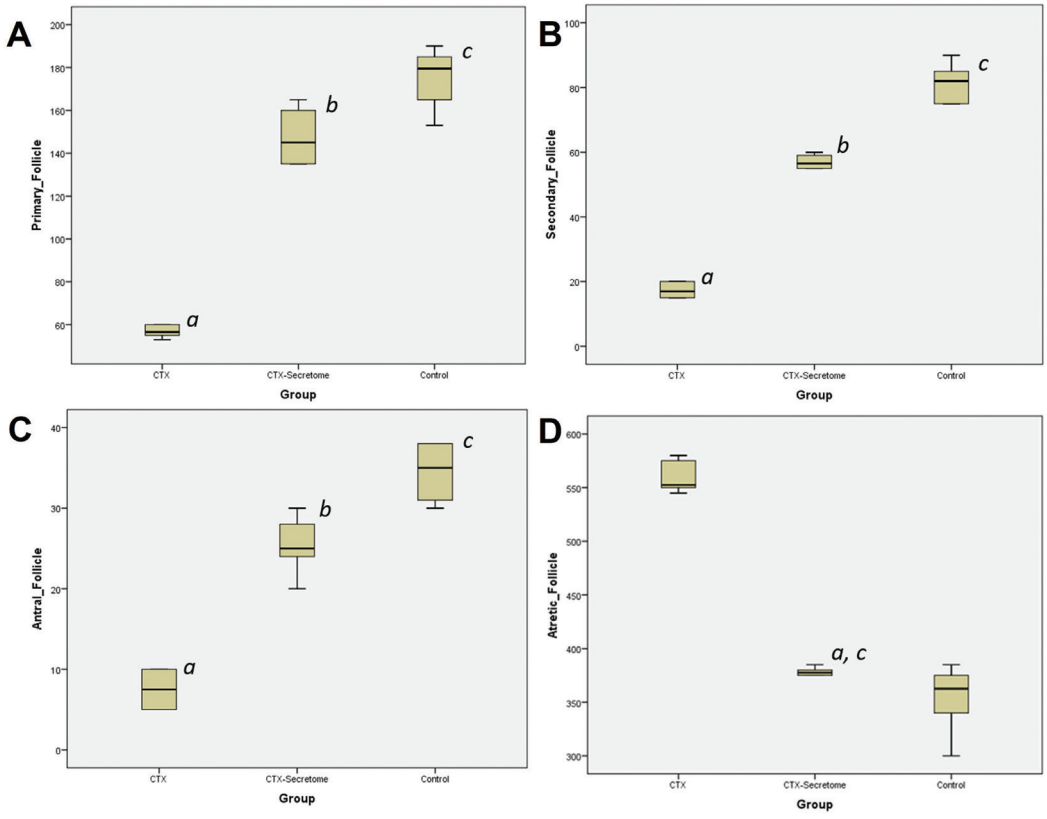

Figure 3. Boxplot graph of (A) primary follicle, (B) secondary follicle, (C) antral follicle, (D) atretic follicle. Data are presents as mean ± SD (n=6 per group). a = p<0.06 vs control, b = p<0.05 vs CTX, c = p<0.05 vs. CTX+secretome

CTX: Cyclophosphamide, SD: Standard deviation

than in the CTX group (9.98±2.8 vs. 5.92±0.74; p<0.05). The control group differed significantly from the CTX-secretome result, with values of 11.78±3.51 ng/mL and 9.98±2.8 ng/mL, respectively.

Discussion

This study established the POI mouse model based on the preceding experiment that used several doses of CTX. The optimal dose that did not cause more than a dropout rate in the mouse population was 50 mg/kg on the first day, followed by 4 mg of CTX for the next 13 days. The preceding experiment was performed to ensure that the animals survived to undergo the subsequent experiment, and it showed an aging effect compared with the control group.

In this study, we found that ovarian weight in the CTX group was significantly lower than the control and CTX-secretome groups. Histological examination also revealed significant changes in the CTX group: Decreased numbers of primary, secondary, and antral follicles and increased numbers of atretic follicles. These findings are consistent with the studies by Song et al.[6] and Pouladvand et al.[16], who reported that CTX promotes follicular atresia by inducing granulosa cell apoptosis, overstimulating dormant primordial follicles (PMFs) via the PTEN/Akt/FOXO3 and mTOR pathways, and generating DNA double-strand breaks that overactivate the ATM–CHK2-Tap63 pathway, leading to apoptosis of PMF oocytes[15]. Another mechanism underlying CTX-induced POI is excessive oxidative stress, which causes mitochondrial dysfunction and upregulates pro-apoptotic and pro-inflammatory mediators such as nuclear factor kappa, tumor necrosis factor alpha, cyclooxygenase-2, and inducible nitric oxide synthase, thereby amplifying cellular injury and death[16]. In addition, CTX disrupts the endocrine balance by reducing circulating levels of estrogen and progesterone, while increasing circulating levels of follicle-stimulating hormone and luteinizing hormone. This hormonal imbalance accelerates follicular depletion and atresia, ultimately reducing the number of mature follicles in the ovary[9,16,17]. At the tissue level, CTX also impaired angiogenesis (resulting in reduced ovarian blood supply) and induced fibrosis within the ovarian cortex, further impairing follicular survival and ovarian function[6].

Secretome treatment resulted in partial restoration of ovarian morphology and function. Ovarian weight increased after secretome injection compared with CTX, reflecting the increased number of primary, secondary, and antral follicles in the ovary. This recovery correlates with a significant increase in AMH levels in the CTX-secretome group compared with the CTX group, indicating better preservation of the follicular pool. Mechanistically, FGF signalling—especially that mediated by FGF2, FGF8, FGF18, and FGF21—could restore PI3K/AKT activity and counteract FOXO3-driven dormancy, thereby rescuing granulosa cell proliferation and angiogenesis[15]. Simultaneously, transforming growth factor-beta/SMAD signalling may attenuate the overactivation of the ATM-CHK2-TAp63 pathway, essential for balancing oocyte elimination and survival. Moreover, FGF21 induces GC proliferation and estradiol production and activates crosstalk between the PI3K/AKT and mTOR pathways. Secretome-derived factors may restore homeostasis within the ovarian microenvironment, mitigating CTX-induced injury at multiple checkpoints[15].

However, the result for the CTX-secretome group still shows substantial differences from the control group. It can serve as the basis for a subsequent study evaluating different doses of secretome to achieve an optimal value approximating the normal value.

Study Limitations

This research has several limitations. We did not incorporate biochemical or molecular markers that would have further strengthened the secretome in POI. The present study focuses on an experimental approach to demonstrate morphological and endocrine recovery following secretome therapy. Furthermore, a single dose of secretome administered exclusively via the intraperitoneal route limits conclusions regarding dose optimization and may overlook differences associated with alternative routes of administration. The lack of long-term fertility assessments, such as mating success and offspring viability, also limits the study. Nevertheless, this study offers novel insights and underscores secretome-based therapy as a promising strategy to preserve ovarian reserve against chemotherapy-induced damage.

Conclusion

The POI condition was induced in normal mice by CTX injection, and secretome injection significantly improved follicle number, ovarian weight, and AMH level. However, the result has not yet reached the normal value observed in the control group. It can be concluded that the secretome improved ovarian quality compared with the POI group. However, it had not yet returned to the quality of the normal ovary. Notably, the following study requires testing different doses of secretomes to determine the optimal dose.

Ethics

Ethics Committee Approval: The Research Ethics Committee, Faculty of Medicine, Universitas Udayana (approval number: 2713/UN14.2.2.VII.14/LT/2024, date: 11.11.2024).

Informed Consent: Retrospective study.

Footnotes

Authorship Contributions

Surgical and Medical Practices: S.K., I.G.E.W., I.N.G.B., Concept: S.K., I.G.E.W., I.W.P.S.Y., Design: I.G.E.W., I.W.P.S.Y., Data Collection or Processing: S.K., I.W.P.S.Y., I.N.G.B., Analysis or Interpretation: S.K., I.W.P.S.Y., I.N.G.B., Literature Search: S.K., I.W.P.S.Y., Writing: S.K., I.G.E.W., I.N.G.B.

Conflict of Interest: No conflict of interest was declared by the authors.
Financial Disclosure: The authors declared that this study received no financial support.

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Clinical Investigation / Araştırma

DOI: 10.4274/tjod.galenos.2025.29938

Turk J Obstet Gynecol 2025;22(4):321-8

Antimicrobial resistance and multidrug resistance patterns of uropathogens isolated from pregnant women with asymptomatic bacteriuria

Asemptomatik bakteriüri olan gebelerden izole edilen üropatojenlerin antimikrobiyal direnç ve çoklu ilaç direnç profilleri

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Abstract

Objective: This study aimed to evaluate the prevalence of asymptomatic bacteriuria (ASB) in the second half of pregnancy, identify causative microorganisms, and assess their antimicrobial resistance and multidrug resistance (MDR) patterns in Kırşehir, Türkiye.

Materials and Methods: Between April-December 2024, 182 pregnant women without urinary tract infection symptoms were screened at Kırşehir Training and Research Hospital. Midstream urine samples were cultured, and bacterial isolates were identified and tested for antimicrobial susceptibility using the BD Phoenix™ automated system. Data were interpreted according to EUCAST 2024 criteria.

Results: ASB prevalence was 37.36%. *Escherichia coli* (51.47%) was the most common pathogen, followed by *Candida* spp. (17.65%), *Klebsiella pneumoniae* (8.82%), and *Streptococcus agalactiae* (7.36%). In Gram-negative isolates, the highest resistance was to ampicillin (72.7%), cefazolin (43.2%), and amoxicillin-clavulanate (40.9%), with universal susceptibility to amikacin, carbapenems, and nitrofurantoin. Gram-positive isolates showed the highest resistance to moxifloxacin and tetracycline (41.7% each). MDR was detected in 20% of *Escherichia coli*, 16.7% of *Klebsiella pneumoniae*, 60% of *Streptococcus agalactiae*, and 66.6% of *Staphylococcus epidermidis* isolates.

Conclusion: ASB prevalence during second half of pregnancy was high, and a significant proportion of pathogens demonstrated MDR. The findings highlight the necessity of culture-based diagnosis and region-specific empirical therapy. High resistance to ampicillin and trimethoprim–sulfamethoxazole suggests that empirical protocols should be updated according to local antibiograms. Strengthening antibiotic stewardship and expanding routine ASB screening are critical to reducing maternal–fetal complications.

Keywords: Asymptomatic bacteriuria, pregnancy, antimicrobial resistance, multidrug resistance

Öz

Amaç: Bu çalışma, gebeliğin ikinci yarısında asemptomatik bakteriüri (ASB) prevalansını değerlendirmek, etken mikroorganizmaları belirlemek ve bunların antimikrobiyal direnç ile çoklu ilaç direnci (MDR) paternlerini incelemek amacıyla Kırşehir, Türkiye'de gerçekleştirilmiştir.

PRECIS: Asymptomatic bacteriuria in pregnancy showed significant prevalence with *Escherichia coli*, *Klebsiella pneumonia*, *Streptococcus agalactia* and *Candida* spp. predominance and high multidrug resistance rates, underscoring the need for culture-based diagnosis and region-specific antibiotic strategies.

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Received/Geliş Tarihi: 18.08.2025 **Accepted/Kabul Tarihi:** 22.10.2025 **Epub:** 27.10.2025 **Publication Date/Yayınlanma Tarihi:** 02.12.2025

Cite this article as: Öztürk C, Kara M, Akyol R, Bolacalı M. Antimicrobial resistance and multidrug resistance patterns of uropathogens isolated from pregnant women with asymptomatic bacteriuria. Turk J Obstet Gynecol. 2025;22(4):321-8

Gereç ve Yöntemler: Nisan-Aralık 2024 tarihleri arasında, Kırşehir Eğitim ve Araştırma Hastanesi'nde üriner sistem enfeksiyonu semptomu olmayan 182 gebe kadın taranmıştır. Orta akım idrar örnekleri kültüre alınmış, bakteri izolatları tanımlanmış ve antimikrobiyal duyarlılık testleri BD Phoenix™ otomatize sistemi ile yapılmıştır. Veriler, EUCAST 2024 kriterlerine göre yorumlanmıştır.

Bulgular: ASB prevalansı %37,36 olarak bulunmuştur. En sık patojen *Escherichia coli* (%51,47) olup, bunu *Candida* spp. (%17,65), *Klebsiella pneumoniae* (%8,82) ve *Streptococcus agalactiae* (%7,36) izlemiştir. Gram-negatif izolatlarda en yüksek direnç ampisilin (%72,7), sefazolin (%43,2) ve amoksisilin-klavulanata (%40,9) karşı belirlenmiş; tüm izolatların amikasin, karbapenemler ve nitrofurantoine duyarlı oldukları saptanmıştır. Gram-pozitif izolatlarda en yüksek direnç moksifloksasin ve tetrasikline (%41,7) karşı görülmüştür. MDR oranları *Escherichia coli* izolatlarında %20, *Klebsiella pneumoniae*'de %16,7, *Streptococcus agalactiae*'de %60 ve *Staphylococcus epidermidis*'de %66,6 olarak belirlenmiştir.

Sonuç: Gebeliğin ikinci yarısında ASB prevalansı yüksek olup, patojenlerin önemli bir kısmı MDR göstermiştir. Bulgular, kültür bazlı tanının ve bölgeye özgü ampirik tedavi yaklaşımlarının önemini ortaya koymaktadır. Ampisilin ve trimethoprim-sülfametoksazole karşı yüksek direnç, ampirik tedavi protokollerinin yerel antibiyogramlara göre güncellenmesi gerektiğini göstermektedir. Antibiyotik yönetiminin güçlendirilmesi ve rutin ASB taramalarının yaygınlaştırılması, maternal-fetal komplikasyonların azaltılması açısından kritik öneme sahiptir.

Anahtar Kelimeler: Asemptomatik bakteriüri, gebelik, antimikrobiyal direnç, çoklu ilaç direnci

Introduction

Asymptomatic bacteriuria (ASB) is defined as the growth of a single uropathogen at concentrations typically ≥10^5 colony-forming unit (CFU)/mL in a properly collected midstream urine sample, without the presence of urinary tract infection symptoms[1]. During pregnancy, hormonal and anatomical changes, particularly the smooth muscle-relaxing effect of progesterone, cause urinary tract dilatation and decreased bladder tone, thereby increasing urinary stasis and creating a favorable environment for bacterial colonization[2,3].

In pregnant women, untreated ASB increases the risk of acute pyelonephritis by approximately four to tenfold, potentially leading to serious complications such as premature birth, low birth weight, preeclampsia, and even maternal sepsis[4-6]. The incidence of pyelonephritis during pregnancy has been observed to rise to as high as 20-40% when ASB is left untreated[1,7]. For this reason, international authorities such as the Infectious Diseases Society of America, the American College of Obstetricians and Gynecologists, the World Health Organization, emphasize the importance of screening with urine culture at least once during early pregnancy and treating positive cases with appropriate antibiotics[1,8-10].

The prevalence of ASB among pregnant women varies depending on geographic region, socioeconomic status, hygiene practices, access to healthcare services, sampling methods, and diagnostic criteria, with global rates reported between 2% and 15%[11-13]. In certain regions such as Africa and South Asia, rates exceeding 20% have been reported[12-15]. Among causative microorganisms, *Escherichia coli* (*E. coli*) is the most isolated and identified, followed by *Klebsiella pneumoniae* (*K. pneumoniae*), *Streptococcus agalactiae* (*S. agalactiae*), *Enterococcus faecalis* and, less commonly, fungal species[16,17].

Recently, the rise of antimicrobial resistance has emerged as a significant public health concern in the management of ASB during pregnancy. In particular, high resistance rates to ampicillin and amoxicillin suggest that nitrofurantoin, amoxicillin-clavulanate, and cephalosporins may be more reliable empirical treatment options[1,9,18]. However, as resistance patterns exhibit regional variation, regular updates of local antibiogram data are recommended for each healthcare setting[19,20].

Although several studies on ASB in pregnant women have been conducted in various regions of Türkiye, no published research has specifically addressed the prevalence, causative microorganisms, and antibiotic resistance patterns of ASB in pregnant women in Kırşehir province. This study was therefore designed to determine the prevalence of ASB among pregnant women attending Kırşehir Training and Research Hospital, to identify the causative microorganisms and their associations with maternal age groups, and to evaluate antibiotic resistance rates. The findings are expected to contribute to regional resistance data and provide guidance for clinical practice in managing ASB during pregnancy.

Materials and Methods

Collection and Transportation of Specimens to the Laboratory

Urine samples obtained from pregnant women attending the Obstetrics and Gynecology Outpatient Clinic of Kırşehir Training and Research Hospital for antenatal care were delivered under aseptic conditions to the Medical Microbiology Laboratory. A total of 182 clinical specimens collected between April 2024 and December 2024 were examined. The study was approved by Kırşehir Ahi Evran University Health Sciences Scientific Research Ethics Committee (decision no: 2024-08/55, dated: 02.04.2024), and written informed consent was obtained from all participants.

The inclusion criteria were as follows: pregnant women in the second half of gestation who had no symptoms of urinary infection (such as frequency, dysuria, flank pain, or fever), had not used antibiotics in the previous 14 days, had no history of urological anomalies or chronic kidney disease, and were willing to participate in the study.

All specimens were collected using the midstream clean-catch technique under aseptic conditions[21]. Prior to sampling, participants were instructed on appropriate genital hygiene procedures. The collected samples were placed in sterile screw-capped containers and quickly transported to the Medical Microbiology Laboratory of Kırşehir Training and Research Hospital. Microbiological analyses were performed

within a maximum of two hours after sample arrival at the laboratory.

Microbiological Examination and Antimicrobial Susceptibility

All specimens delivered to the microbiology laboratory under aseptic conditions were inoculated onto 5% sheep blood agar and eosin-methylene blue agar using standard microbiological techniques. The inoculated plates were incubated aerobically at 35-37 °C for 18-24 hours. Growth of a microorganism at a concentration of $\geq 10^5$ CFU/mL was considered indicative of ASB[1]. A pure culture was obtained. Preliminary identification of pure culture isolates was performed using Gram staining, catalase, oxidase, and coagulase tests. Bacterial identification and antimicrobial susceptibility testing were subsequently carried out with the BD Phoenix™ automated system. Identification and antibiotic susceptibility tests were performed according to the manufacturer's instructions.

Antimicrobial susceptibility data were interpreted according to the 2024 criteria of the European Committee on Antimicrobial Susceptibility Testing[22].

Regarding Gram-negative bacteria, susceptibility testing was performed for the following antibiotics: amikacin, ciprofloxacin, gentamicin, levofloxacin, trimethoprim-sulfamethoxazole, ampicillin, tigecycline, ceftazidime, ceftriaxone, cefuroxime, cefazolin, imipenem, meropenem, piperacillin-tazobactam, amoxicillin-clavulanate, ertapenem, cefixime, nitrofurantoin, and tobramycin. With respect to Gram-positive bacteria, the tested antibiotics included amikacin, vancomycin, ciprofloxacin, daptomycin, erythromycin, fusidic acid, gentamicin, levofloxacin, linezolid, moxifloxacin, oxacillin, penicillin G, trimethoprim-sulfamethoxazole, tetracycline, clindamycin, and rifampin.

Statistical Analysis

All data were analyzed using SPSS version 26.0. The chi-square test or Fisher's exact test was applied for categorical variables, and statistical significance was reached when $p<0.05$.

Results

Prevalence of ASB and Age Groups

A total of 182 pregnant women in the second half of pregnancy were included in this research. The mean age of the participants was 29.7±7.0 (minimum: 18 - maximum: 44) years, and for statistical analysis, the age distribution was divided into three groups (Table 1). Significant bacteriuria was detected in 37.36% of the total samples. In the remaining 62.84%, either no bacterial growth was observed or the growth did not meet the diagnostic criteria. No statistically significant relationship was found between age groups and ASB positivity ($p>0.05$, Table 1).

In the 68 ASB-positive samples, a total of 10 different bacterial species and *Candida* spp. were isolated. The most frequently identified pathogen was *E. coli*, detected in 51.47% (n=35) of all positive samples. This was followed by *Candida* spp. (17.65%), *K. pneumoniae* (8.82%), and *S. agalactiae* (7.36%) (Table 2, Figure 1).

Antimicrobial Susceptibility

When ASB-positive samples were evaluated by species, *E. coli* (n=35) isolates showed the highest resistance rates to ampicillin (71%), followed by trimethoprim-sulfamethoxazole (41.2%), amoxicillin-clavulanate (37.1%), ceftazidime (25.7%), and ceftriaxone (20%). *K. pneumoniae* (n=6) isolates exhibited intrinsic resistance to ampicillin and showed resistance rates of 83.3% to piperacillin-tazobactam, 66.7% to amoxicillin-clavulanate, and 66.7% to cefazolin. In *S. agalactiae* (n=5) isolates, resistance was detected against erythromycin (40%), levofloxacin (40%), moxifloxacin (60%), tetracycline (60%), gentamicin (40%), and chloramphenicol (40%) (Figure 2).

In Gram-negative isolates, the highest resistance was observed against ampicillin (72.72%), followed by cefazolin (43.18%), amoxicillin-clavulanate (40.9%), and trimethoprim-sulfamethoxazole (34.9%). All isolates were found to be susceptible to amikacin, imipenem, nitrofurantoin, and meropenem (Table 3, Figure 3).

When examining the antibiotic resistance patterns of Gram-positive bacteria, the highest resistance rates were found against moxifloxacin and tetracycline, each at 41.67%. This was followed by levofloxacin at 33.33% and gentamicin at 25%.

Table 1. Distribution of ASB positivity and age groups

Age	Healthy n (%)	ASB n (%)	Test statistic value, p-value
18-25	34 (29.8)	24 (35.3)	χ^2=2.023 p=0.364
26-33	49 (43.0)	22 (32.4)	
34 and above	31 (27.2)	22 (32.4)	

χ^2: Pearson chi-square, p=0.364, ASB: Asymptomatic bacteriuria

Table 2. Microorganism distribution at the species level in asymptomatic bacteriuria positive samples

Microorganism	%
***Candida* spp.**	**17.65**
Enterobacter aerogenes	1.47
Enterococcus faecalis	1.47
Escherichia coli	**51.47**
Klebsiella pneumoniae	**8.82**
Lactobacillus spp.	1.47
Proteus mirabilis	2.94
Staphylococcus aureus	1.47
Staphylococcus epidermidis	4.41
Streptococcus agalactia	**7.36**
Streptococcus pyogenes	1.47

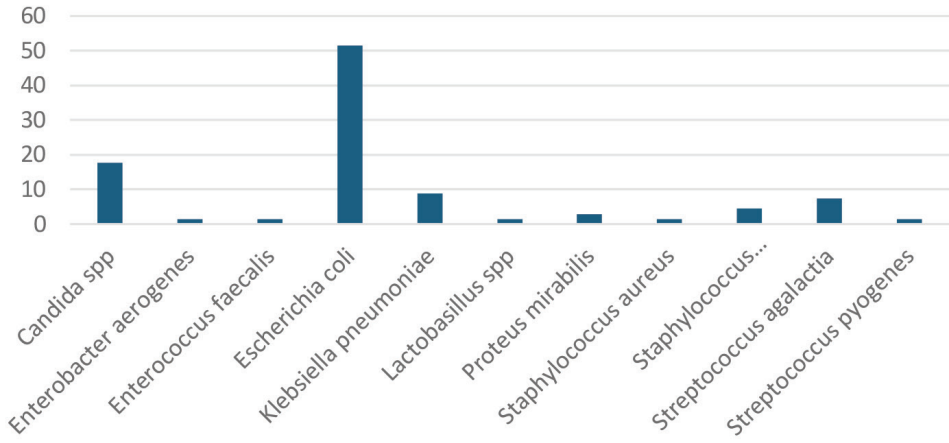

Figure 1. Microorganism distribution at the species level in asymptomatic bacteriuria positive samples

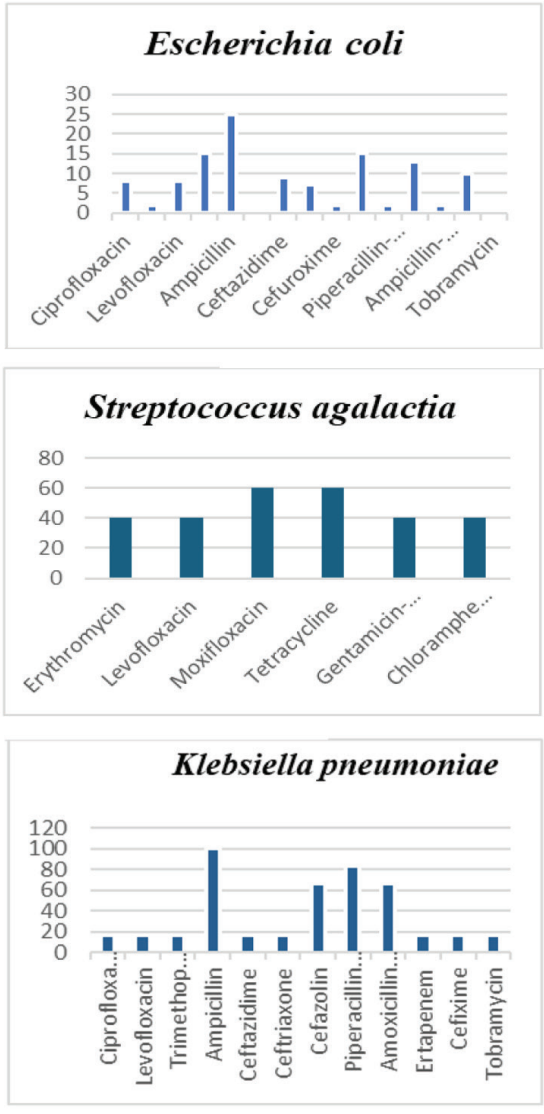

Figure 2. Antimicrobial resistance distribution of pathogens frequently isolated in the diagnosis of asymptomatic bacteriuria during pregnancy

Among Gram-positive bacteria, resistance to ciprofloxacin, fusidic acid, oxacillin, chloramphenicol, and erythromycin was observed at a rate of 16.67%, while rifampin, trimethoprim-sulfamethoxazole, penicillin G, and clindamycin showed lower resistance rates of 8.33% No resistance was detected to vancomycin, teicoplanin, daptomycin, linezolid, amikacin, or gentamicin (Table 4, Figure 4).

Multidrug Resistance in Bacteria

Examination of the antibiotic classes tested against bacterial isolates from ASB positive samples revealed that some were resistant to three or more classes, qualifying them as multidrug resistance (MDR) organisms. In our study, among Gram-negative bacteria, MDR was detected in 20% of *E. coli* isolates and 16.7% of *K. pneumoniae* isolates. In Gram-positive bacteria, resistance rates were notably higher; 60% of *S. agalactiae* isolates and 66.6% of *S. epidermidis* isolates were resistant to at least three different antibiotic classes (Figure 5).

The chi-square test revealed no statistically significant association between bacterial species and MDR (X^2=5.99, p=0.071), indicating that the variables may be independent. Accordingly, the observed frequencies did not differ significantly from the expected values. The detailed results of the chi-square analysis are presented in Table 5.

Discussion

This study was conducted to reveal the prevalence of ASB in the second half of pregnancy in Kırşehir province, identify the causative microorganisms, and evaluate their antimicrobial resistance patterns. Given the absence of a previous comprehensive investigation at the regional level, our findings aim to contribute both locally and nationally by updating epidemiological data and guiding clinicians toward effective antibiotic therapy.

In our investigation, the prevalence of ASB in the second half of pregnancy was found to be 37.36%. These findings are consistent with certain studies conducted in Türkiye.

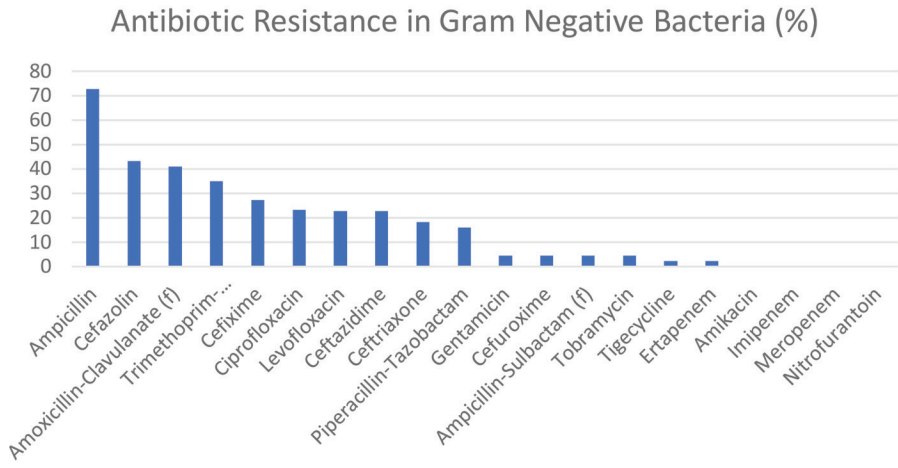

Figure 3. Selected antibiotic susceptibility rates of Gram-negative isolates

Table 3. Antibiotic susceptibility results of Gram-negative isolates

Antibiotics	Resistance	n (%)
Ampicillin	32	72.7
Cefazolin	19	43.2
Amoxicillin-clavulanate	18	40.9
Trimethoprim-sulfamethoxazole	15	34.9
Cefixime	12	27.3
Ciprofloxacin	10	23.3
Levofloxacin	10	22.7
Ceftazidime	10	22.7
Ceftriaxone	8	18.2
Piperacillin-tazobactam	7	15.9
Gentamicin	2	4.5
Cefuroxime	2	4.5
Tobramycin	2	4.5
Tigecycline	1	2.3
Ertapenem	1	2.3
Amikacin	0	0
Imipenem	0	0
Meropenem	0	0
Nitrofurantoin	0	0

Aktün et al.[23] reported a prevalence of 36.5%, while Efe and Kurdoğlu[24] reported 35.4%. However, lower prevalence rates (4-20%) have been reported in other regions, a variation likely attributable to geographical differences, socioeconomic conditions, hygiene practices, and discrepancies in laboratory diagnostic methods[3,25].

In our study, the most frequently isolated pathogen was *E. coli* (51.47%), followed by *Candida* spp. (17.65%), *K. pneumoniae* (8.82%), and *S. agalactiae* (7.36%). This distribution aligns with previous reports indicating *E. coli* as the predominant etiological agent in ASB during pregnancy[1,11]. The relatively higher proportion of *Candida* spp. in our cohort compared to some earlier studies may reflect local epidemiological characteristics, patient-specific factors such as recent antibiotic exposure or gestational diabetes, or laboratory detection practices.

When analyzed by antimicrobial susceptibility, *E. coli* isolates demonstrated the highest resistance rates to ampicillin (71%), trimethoprim-sulfamethoxazole (41.2%), and amoxicillin-clavulanate (37.1%), with moderate resistance to ceftazidime (25.7%) and ceftriaxone (20%). These results are consistent with national and international data, which report persistently high resistance to ampicillin and increasing resistance to β-lactamase inhibitor combinations[20,26]. Importantly, all *E. coli* isolates in our study retained susceptibility to aminoglycosides (amikacin), carbapenems, and nitrofurantoin, suggesting that these agents remain viable options for empirical therapy in our setting.

The *K. pneumoniae* isolates demonstrated notably high resistance to piperacillin-tazobactam (83.3%) and amoxicillin-clavulanate (66.7%). Among Gram-positive pathogens, *S. agalactiae* exhibited marked resistance to moxifloxacin (60%) and tetracycline (60%), a finding that may have implications for antenatal prophylaxis strategies[10].

When MDR rates were examined, it was determined that 20% of *E. coli* isolates, 16.7% of *K. pneumoniae* isolates, 60% of *S. agalactiae* isolates, and 66.6% of *S. epidermidis* isolates had MDR. The particularly higher MDR rates among Gram-positive bacteria represent a clinically significant finding, as they may limit therapeutic options and increase the risk of maternal-fetal complications[6,20].

These results underscore the importance of routine ASB screening during pregnancy and the implementation of culture-based antibiotic therapy. The high resistance rates observed for commonly used agents such as ampicillin and trimethoprim-sulfamethoxazole suggest that empirical treatment protocols should be revised in light of local resistance patterns[5].

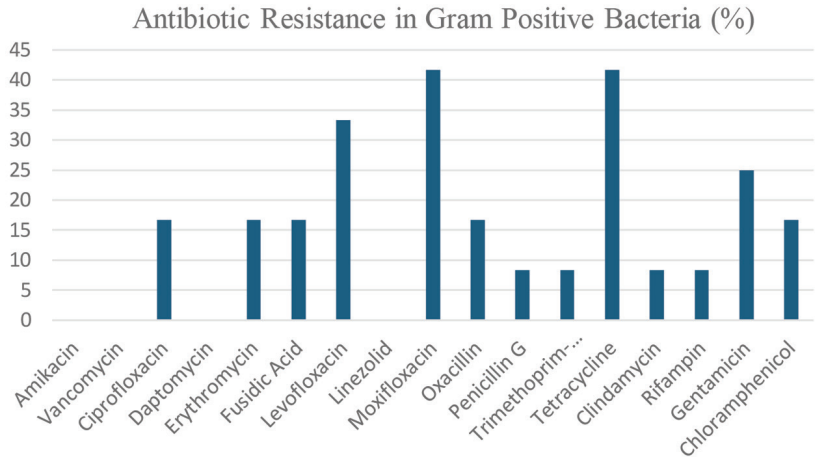

Figure 4. Susceptibility rates of Gram-positive isolates to selected antibiotics

Table 4. Antibiotic susceptibility results of Gram-positive isolates

Antibiotics	Number of isolate (n)	Resistance rate (%)
Rifampin	1	8.3
Moxifloxacin	5	41.7
Tetracycline	5	41.7
Ciprofloxacin	2	16.7
Fusidic acid	2	16.7
Oxacillin	2	16.7
Chloramphenicol	2	16.7
Gentamicin	3	25.0
Levofloxacin	4	33.3
Erythromycin	2	16.7
Trimethoprim-sulfamethoxazole	1	8.3
Penicillin G	1	8.3
Clindamycin	1	8.3
Vancomycin	0	0
Teicoplanin	0	0
Daptomycin	0	0
Linezolid	0	0
Amikacin	0	0

Table 5. The relationship between bacterial species and multidrug resistance

	MDR + n (%)	MDR – n (%)	Test statistic value, p-value
E. coli	7 (20.0%)	28 (80.0%)	X^2:=5.99*; p=0.071
K. pneumoniae	1 (16.7%)	5 (83.3%)	
S. agalactiae	3 (60.0%)	2 (40.0%)	
S. epidermidis	2 (66.7%)	1 (33.3%)	
*: Fisher's exact test, MDR: Multidrug resistance			

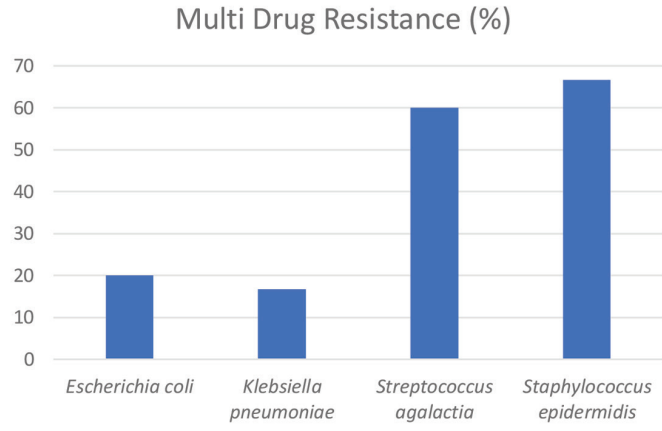

Figure 5. Distribution of multidrug-resistant bacterial isolates

Study Limitations

Expanding the sample size, extending the study period, and performing multicenter comparative investigations would provide more comprehensive insights into the prevalence and antimicrobial resistance patterns of ASB in second half of pregnancy.

Conclusion

In the study, the prevalence of ASB in the second half of pregnancy is high, and a substantial proportion of the causative agents exhibit multidrug resistance. In particular, *E. coli* and *K. pneumoniae* isolates demonstrated high resistance to ampicillin, amoxicillin–clavulanate, and cephalosporins, whereas *S. agalactiae* showed significant resistance to fluoroquinolones and tetracycline. Therefore, diagnosis of ASB during pregnancy

should always be guided by urine, and antimicrobial susceptibility testing, and empirical treatment should be based on local resistance profiles.

Moreover, the high MDR rates highlight the need to reassess antibiotic use policies in pregnant populations and to strengthen strategies aimed at combating antimicrobial resistance. In this context, expanding routine ASB screening and tailoring treatment protocols according to updated regional resistance data are of critical importance for the health of both mothers and their infants.

Ethics

Ethics Committee Approval: The study was approved by Kırşehir Ahi Evran University Health Sciences Scientific Research Ethics Committee (decision no: 2024-08/55, dated: 02.04.2024).

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

Footnotes

Authorship Contributions

Concept: C.Ö., M.K., Design: C.Ö., M.K., Data Collection or Processing: M.K., R.A., Analysis or Interpretation: C.Ö., R.A., M.B., Literature Search: C.Ö., M.K., R.A., M.B., Writing: C.Ö., M.B.

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

Financial Disclosure: The authors declared that this study received no financial support.

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