



The effect of tinzaparin sodium and leuprolide acetate in an experimental mouse model of endometriosis: The rol of the WNT/beta-catenin pathway

Endometriyozis deneysel fare modelinde tinzaparin sodyum ve löprolid asetatin etkisi: WNT/beta-katenin yolaklarının rolü

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Abstract

Objective: Endometriosis is a benign condition driven by estrogen and inflammation in which endometrial-like tissue develops in ectopic locations. We aimed to determine whether non-steroidal agents acting on the WNT/beta-catenin signaling axis could provide therapeutic benefit in this disease.

Materials and Methods: Forty adult female mice underwent surgical creation of endometriotic implants and were then distributed into five experimental arms: untreated controls, early leuprolide (leup1d), early tinzaparin (tnz1d), delayed leuprolide (leup7d), and delayed tinzaparin (tnz7d). Early treatment groups received drug treatment beginning at postoperative hour 24, whereas delayed groups began treatment on postoperative day 8. At two weeks post-surgery, lesions were harvested for RNA extraction and transcript profiling. Tissues were also processed for hematoxylin-eosin staining with semi-quantitative grading. Immunostaining was performed using antibodies against HIF1a and WNT2.

Results: The tnz7d group exhibited decreased inflammatory markers, while the leup7d group displayed reduced epithelial content; both changes resulted in lower disease severity scores. WNT2 and HIF1a immunostaining revealed greater reductions in score in the tnz7d group compared with controls and other treatment arms, but these differences were not statistically significant.

Conclusion: Further investigation is warranted to determine how tinzaparin sodium and leuprolide acetate modulate the WNT/β-catenin axis for the management of endometriosis.

Keywords: Endometriosis, tinzaparin sodium, leuprolide acetate, WNT/beta-catenin pathway, immunohistochemistry

PRECIS: This study investigates effects of tinzaparin sodium and leuprolide acetate on surgically induced endometriosis in mice, focusing on their impact on the WNT/beta-catenin pathway through gene expression, histopathological, immunohistochemical analyses.

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Öz

Amaç: Endometriozis, iyi huylu, östrojen bağımlı bir enflamatuvar hastalık olup, ektopik endometriyal implantlarla karakterizedir. Bu çalışma, insanlarda endometriozis tedavisi için WNT/beta-katenin yoluna odaklanan yeni nonsteroid ilaçların potansiyelini araştırmayı amaçlamıştır.

Gereç ve Yöntemler: Çalışmaya 40 yetişkin dişi fare dahil edilmiştir. Farelerde cerrahi olarak endometriotik odaklar oluşturulduktan sonra, fareler rastgele beş gruba ayrıldı: 1- Kontrol, 2- Profilaktik leuprolid asetat grubu (leup1d), 3- Profilaktik tinzaparin sodyum grubu (tnz1d), 4- Terapötik leuprolid asetat grubu (leup7d), 5- Terapötik tinzaparin sodyum grubu (tnz7d). Leup1d ve tnz1d gruplarına ameliyat sonrası 24. saatten itibaren profilaktik dozlarda ilaçlar verildi. Leup7d ve tnz7d gruplarına ise ameliyat sonrası 8. günden itibaren terapötik dozlarda ilaçlar verildi. Ameliyat sonrası 14. günde, çıkarılan endometriotik odaklar üzerinde toplam RNA izolasyonu ve gen ekspresyonu analizleri yapıldı. Ek olarak, odaklar hematoksilin ve eozin ile boyandı ve endometriozis için yarı kantitatif olarak puanlandı. HIF1a ve WNT2 için birincil antikörler kullanılarak immünohistokimyasal analiz yapıldı.

Bulgular: Tinzaparin 7 günlük grupta enflamasyonda azalma görüldürken, leuprolid 7 günlük grupta epitel dokusunda azalma görülmüş ve bu da endometriozis skorunda düşüşe yol açmıştır. WNT2 ve HIF1a ile yapılan immünohistokimyasal boyama, kontrol grubu ve diğer gruplara kıyasla tinzaparin 7 günlük grupta endometriozis skorunda daha belirgin bir azalma olduğunu göstermiştir, ancak bu istatistiksel olarak anlamlı değildir.

Sonuç: Endometriozis tedavisinde tinzaparin sodyum ve leuprolid asetatın WNT/beta-katenin yolları üzerindeki etkilerini doğrulamak için daha fazla çalışma yapılması gerekmektedir.

Anahtar Kelimeler: Endometriyozis, tinzaparin sodyum, löprolid asetat, WNT/beta-katenin, immünohistokimya

Introduction

Endometriosis stands as one of the leading benign gynecological disorders, presenting as a persistent, hormone-dependent inflammatory state that leads to extrauterine deposition of glandular and stromal components⁽¹⁾. Affected individuals commonly report cyclic pelvic discomfort, menstrual irregularities, difficulty conceiving, pain during intercourse, urinary symptoms, and bowel complaints. Although the condition is widespread, definitive diagnosis typically lags 7-10 years behind symptom onset due to heterogeneous clinical presentations, complex underlying mechanisms, and insufficient rapid diagnostic options^(2,3).

The precise origins of this condition remain incompletely understood, with evidence pointing to multiple interacting factors. Scientific work has implicated dysregulated WNT/beta-catenin signaling as a contributor to disease pathobiology^(4,5). Although exact pathogenic sequences remain debated, retrograde menstruation followed by peritoneal implantation represents the prevailing explanatory model. Central to this framework are cellular migration and tissue invasion, which are prerequisites for lesion formation⁽¹⁻⁶⁾. Multiple WNT-responsive genes govern cell growth, directional movement, and matrix penetration^(7,8). Current findings indicate that aberrant pathway activation may enhance the migratory and invasive capacity of shed endometrial cells in patients with this condition⁽⁹⁾.

Although classified as non-malignant based on histological criteria, endometriosis exhibits certain biological behaviors resembling cancer. Analogous to neoplastic processes, these lesions demonstrate a capacity for both local extension and distant dissemination⁽¹⁰⁾. Tinzaparin, a low-molecular-weight heparin, has exhibited cytostatic properties by interfering with the WNT/beta-catenin pathway in cellular studies⁽¹¹⁾. Beyond anticoagulation, tinzaparin has shown diverse antitumor activities in preclinical investigations⁽¹²⁾.

Estrogen stands as the sole definitively established factor promoting disease development⁽¹³⁾. Therapeutic objectives

center on alleviating symptoms, arresting progression, and preserving reproductive capacity. Gonadotropin-releasing hormone (GnRH) agonists are first-line pharmacological options that achieve efficacy by inducing a low-estrogen environment. Leuprolide, acting as a GnRH-receptor agonist, suppresses gonadotropin secretion during sustained administration, inducing a reversible ovarian quiescence that mimics menopause⁽¹⁴⁾.

This investigation systematically evaluated the preventive and therapeutic applications of tinzaparin sodium and leuprolide acetate in a surgically induced mouse model of endometriosis, with emphasis on the involvement of WNT/beta-catenin signaling.

Materials and Methods

All procedures received institutional ethical endorsement from Sivas Cumhuriyet University (approval number: 307; date: 03.09.2019) and adhered to established animal welfare principles. Funding was provided by the university's Scientific Research Unit (grant: T879). The study population consisted of 40 sexually mature female mice (body weight 20-25 g) obtained from the institutional animal facility at Sivas Cumhuriyet University. Housing conditions included an ambient temperature of 21±2 °C, relative humidity of 60±5%, and a standard 12-hour photoperiod. Animals had unrestricted access to standard chow and water. Daily environmental monitoring, weekly body weight assessments, and general health evaluations were conducted throughout the study period.

Induction of Endometriosis

The Vernon-Wilson technique was employed, representing the most widely validated approach for establishing peritoneal endometriosis via autologous uterine tissue grafting⁽¹⁵⁾. Anesthesia was induced by intramuscular injection of ketamine (50 mg/mL, Ketalar; Eczacibasi Warner-Lambert, İstanbul, Türkiye) combined with xylazine (20 mg/mL,

Rompun; Bayer, İstanbul, Türkiye), each administered in a volume of 1 mL. Following anesthetic stabilization, the right uterine segment was ligated and surgically removed. A 15 mm tissue specimen was obtained using microsurgical instruments and temporarily stored in isotonic saline. The harvested segment was incised longitudinally and fixed to the peritoneal surface adjacent to the mesenteric vasculature, preserving myometrial architecture. The procedure was completed by abdominal closure.

Following surgery, animals were randomized into five experimental cohorts (n=8 each): (1) Untreated controls; (2) Early leuprolide group (100 µg/day, subcutaneously); (3) Early tinzaparin group (10 mg/kg, subcutaneously), with treatment initiated at 24 hours post-surgery for 14 days; (4) Delayed leuprolide group (100 µg/day, subcutaneously); and (5) Delayed tinzaparin group (10 mg/kg, subcutaneously), with treatment initiated on day 7 post-surgery for 14 days.

Tissue Collection

RNA Isolation and Gene Expression Analysis

Terminal procedures were performed on postoperative day 14 by exsanguination. Repeat laparotomy permitted the identification and careful harvesting of established implants. Specimens were transferred to sterile 1.5 mL tubes containing 1 mL of RNA-preservation solution (Ribo Saver, Gene All, Seoul, Korea) and stored at -80 °C until processing.

Total RNA Isolation and cDNA Collection

Total RNA was extracted using the GeneAll® Hybrid-RTM kit according to the manufacturer's specifications (Cat. no.: 305-101; Lot: 30519L09056; Seoul, Korea). Tissue disruption was performed using magnetic bead homogenization. Purified RNA was reconstituted in 100 µL of nuclease-free water, and the concentration was assessed by spectrophotometry (Denovix DS nanodrop). Specimens yielded 20-40 ng of RNA. Reverse transcription was performed using WizScript™ cDNA Synthesis reagents (South Korea), following the recommended thermal parameters.

Gene Expression Analyses

Transcript quantification was performed using SYBR-based detection chemistry (GeneAll Real Amp™ SYBR master mix, Seoul, Korea) on an Applied Biosystems StepOnePlus platform (USA). SYBR Green served as the reporter dye, with ROX as the passive reference dye. Amplification reactions (10 µL total) contained 2X master mix, 50X ROX, gene-specific primers (10 pmol each), nuclease-free water, and template cDNA. Thermal cycling included initial denaturation (95 °C, 10 min) followed by 40 cycles of denaturation (95 °C, 15 sec) and annealing/extension (60 °C, 60 sec). ACTB served as an endogenous control. Relative quantification was performed using the comparative Ct approach ($2^{-\Delta\Delta Ct}$) to calculate fold-change values.

Histopathological Evaluation

Harvested implants were fixed in 10% phosphate-buffered formalin for 30-36 hours. After standard paraffin processing, 5 µm sections were prepared and subjected to hematoxylin-eosin staining for morphological assessment. Quantitative grading followed established protocols⁽¹⁶⁾. Stromal tissue proportion was determined by averaging coverage across 10 randomly selected high-magnification fields, while glandular density reflected mean gland counts within these fields. Composite scoring employed was as follows: Grade 0: absent stromal or glandular elements; Grade 1: <25% stromal coverage with a single gland; Grade 2: 25-50% stromal coverage with 2-3 glands; Grade 3: >50% stromal coverage with ≥4 glands.

Immunohistochemical Evaluation

Protein expression of WNT2 and HIF1α was assessed immunohistochemically. Deparaffinized sections underwent antigen retrieval via thermal treatment in citrate buffer (0.01 M, pH 6.0) for 10 minutes. Endogenous peroxidase was quenched using 3% hydrogen peroxide. Non-specific binding was blocked with Ultra V reagent (Thermo Fisher Scientific). Primary antibody incubation (WNT2, 1:100, BT-LAB/BT-AS00007; HIF1α, 1:100, BT-LAB/BT-AP00156) was performed overnight at 4 °C. Sequential application of Primary Antibody Enhancer (TL-015-PB; Thermo Fisher Scientific, USA; 20 min) followed by HRP-conjugated polymer (TL-015-PH; Thermo Fisher Scientific, USA; 30 min at room temperature) preceded chromogenic development with DAB substrate. Harris hematoxylin counterstaining facilitated nuclear visualization under bright-field microscopy (Olympus BX50). Immunoreactivity scoring incorporated staining intensity (0=absent, 1=faint, 2=moderate, 3=intense) and percentage of positive cells (0=none, 1=1-10%, 2=11-50%, 3=>50%)⁽¹⁷⁾. Final scores (range 0-9) were derived by multiplying these parameters.

Statistical Analysis

Normality of the data distribution was verified prior to analysis. Statistical analyses were performed using GraphPad Prism 5, and outcomes are reported as mean ± standard error of the mean. Intergroup comparisons for transcript data were performed using one-way ANOVA with Tukey's post-hoc correction. Statistical significance was set at p<0.05.

Results

Drug treatment effects on messenger RNA levels for 12 target genes in control and experimental groups bearing surgical implants are shown in Figure 1.

Histological and Immunohistochemical Staining of Endometriotic Foci

No observable adverse reactions occurred during the experimental period, with body weights remaining

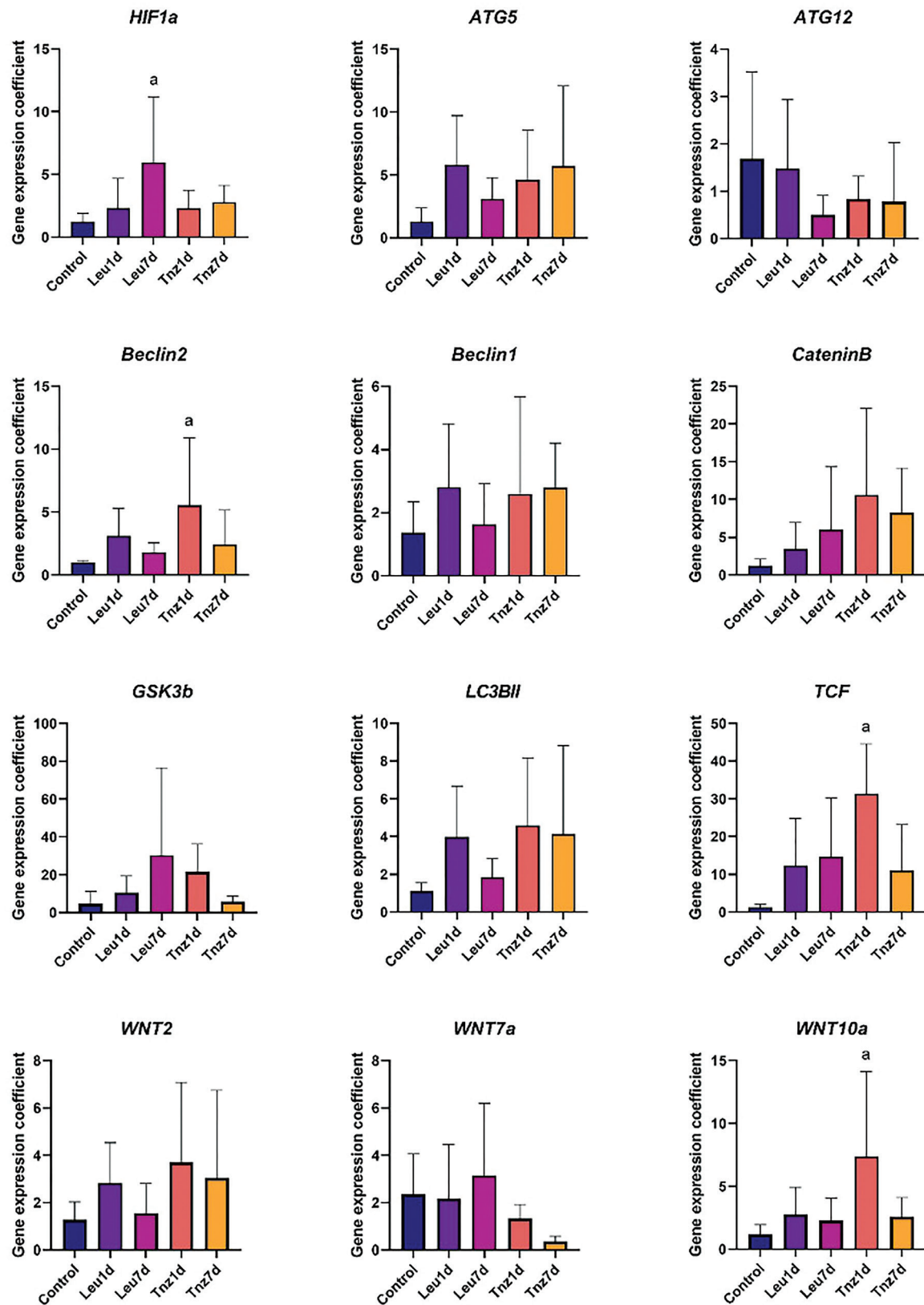


Figure 1. Transcript abundance profiles for control, leu1d, leu7d, tnz1d, and tnz7d groups encompassing WNT2, HIF1a, and related pathway components. Values represent the median and interquartile range

comparable between baseline and endpoint measurements. Representative histological and immunohistochemical preparations showing the expression patterns of WNT2 and HIF1a after treatment with tinzaparin sodium and leuprolide acetate are shown in Figure 2.

Endometriosis Score and Immunohistochemical Staining Score

Comparative analysis revealed that the tnz7d group achieved a statistically significant reduction in disease score, primarily due to decreased inflammatory indices, compared with untreated controls ($p<0.05$; Figure 3). The leup7d group

also demonstrated a significant attenuation of epithelial components compared with controls ($p<0.05$; Figure 3). Immunostaining scores for WNT2 and HIF1a did not differ significantly between either treatment group and the controls ($p>0.05$; Figure 3). Furthermore, no significant inter-drug differences emerged ($p>0.05$, Figure 3).

Discussion

This study systematically evaluated the effects of tinzaparin and leuprolide on surgically-induced murine endometriotic implants by integrated immunohistochemical profiling of WNT2 and HIF1a. Both pharmacological agents demonstrated

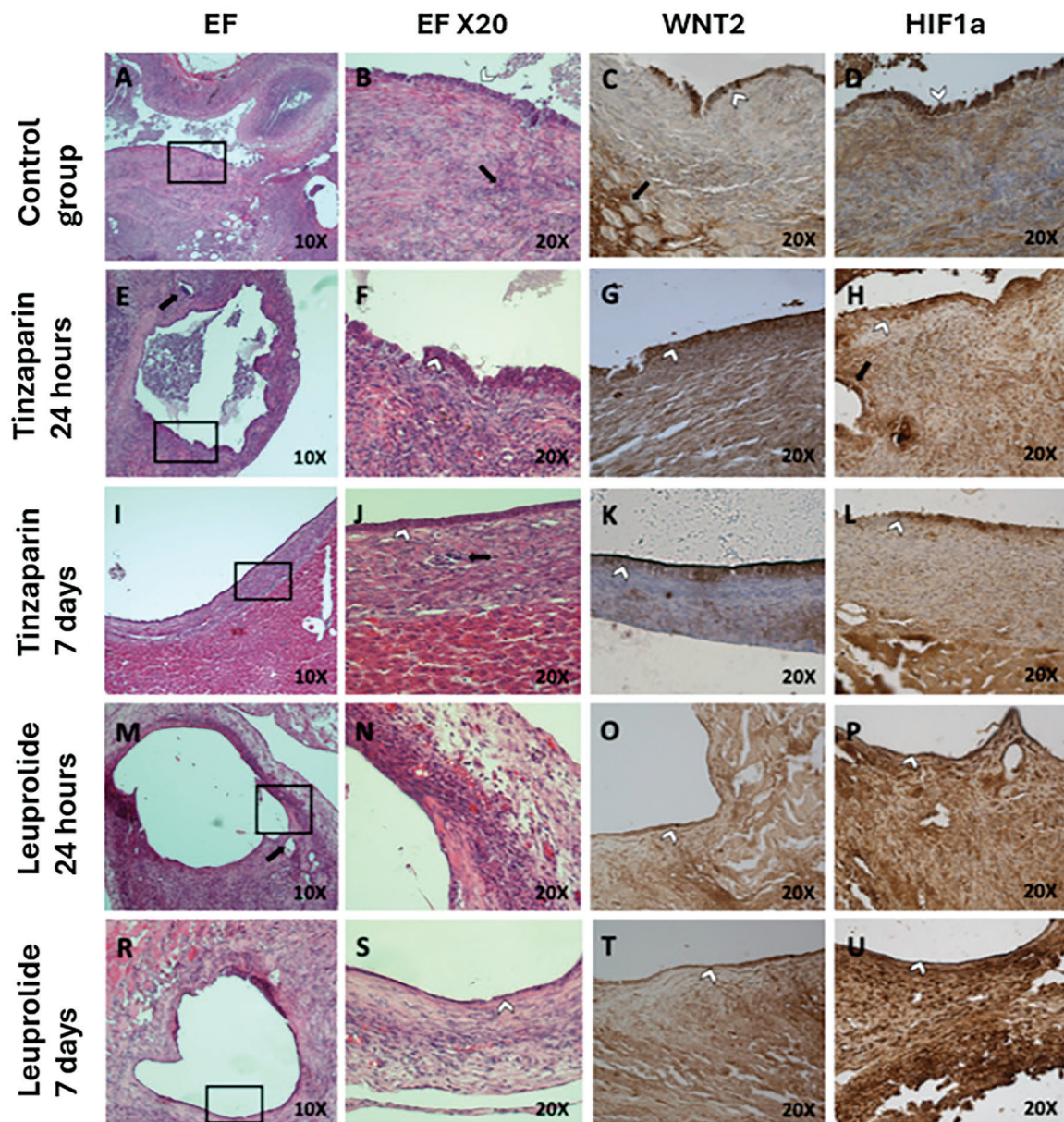


Figure 2. Histological and immunohistochemical findings in endometriotic foci following tinzaparin sodium and leuprolide acetate administration

EF: Endometriotic foci, X20: 20-fold optical magnification

the capacity to attenuate disease severity scores. Although early-treatment groups (initiated on day 1) exhibited numerical reductions in scores relative to untreated controls, these differences were not statistically significant. Notably, the delayed tinzaparin group (tnz7d) produced marked suppression of inflammation, whereas the corresponding leuprolide group (leup7d) significantly reduced epithelial tissue content, and collectively reduced disease burden. Immunostaining analysis revealed that day-7 tinzaparin produced numerically greater score reductions than controls and other treatment arms, although these differences did not reach statistical significance. Leuprolide treatment produced no discernible alteration in immunohistochemical markers relative to controls. The leup7d group exhibited significantly elevated HIF1a transcript levels compared with untreated controls, whereas the tnz1d group showed significant upregulation of BECLIN2/TCF and WNT10a mRNA levels.

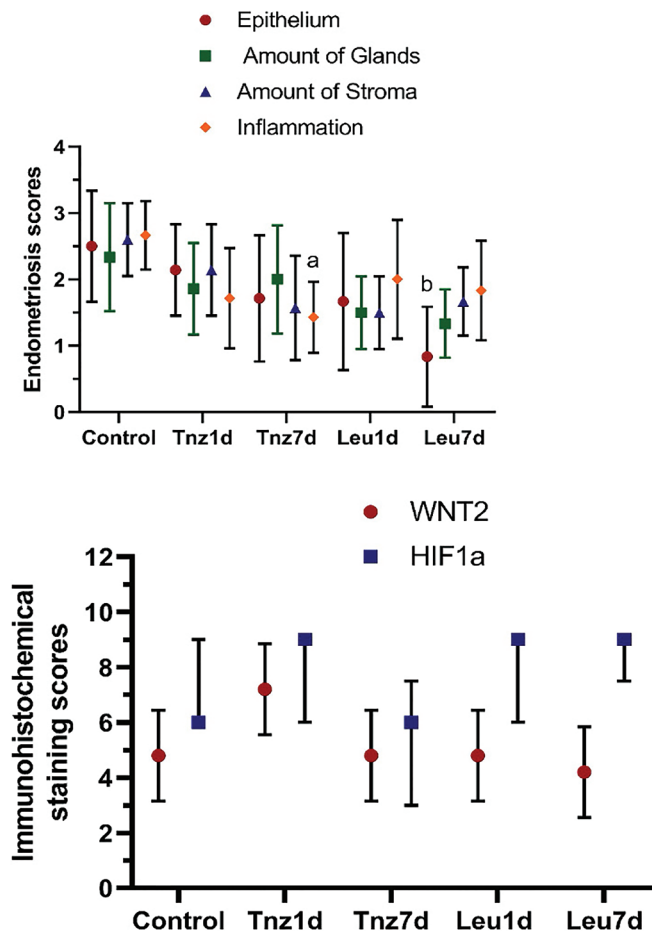


Figure 3. Disease severity scores and immunostaining indices across all experimental groups (control, tnz1d, tnz7d, leup1d, leup7d). Data are shown as median (interquartile range). Inflammatory scores in tnz7d were significantly reduced compared with controls (designated 'a'; $p < 0.05$), and epithelial scores in leup7d were significantly decreased compared with the control and tnz1d groups (designated 'b'; $p < 0.05$)

The etiopathogenesis of endometriosis and optimal therapeutic approaches have generated sustained scientific discourse. Currently available interventions cannot prevent disease onset. Epidemiological data indicate an approximately 6-7-fold increase in disease frequency among first-degree biological relatives of affected individuals compared with background population rates⁽¹⁸⁾. Concordance studies in monozygotic twins reveal heritability estimates approaching 75%⁽¹⁹⁾, substantiating genetic predisposition as a significant determinant of susceptibility.

Despite an unremarkable cellular architecture, endometriotic lesions exhibit several pathobiological features characteristic of malignancy. Paralleling neoplastic behavior, these implants demonstrate a propensity for regional tissue invasion and dissemination to anatomically remote sites. Such lesions exhibit adhesive properties, enabling attachment to and penetration of adjacent structures. Nevertheless, distinguishing features include the absence of a sustained proliferative drive and of cachexia-inducing metabolic perturbations; fatal outcomes are exceptional⁽²⁰⁾. Importantly, endometriosis constitutes an inflammatory condition, and chronic inflammatory states have established associations with elevated malignancy risk.

Selection of tinzaparin derived from encouraging cellular assay data demonstrating cytotoxic efficacy against transformed cells through WNT/beta-catenin pathway interference, supplemented by established clinical safety for thromboembolic prophylaxis across diverse patient populations including pregnant women⁽¹¹⁾. These attributes positioned tinzaparin as a rational candidate for evaluating the involvement of pathways in the pathogenesis of endometriosis. Published literature documents tinzaparin's antimetastatic and antiangiogenic activities and its modulation of the WNT pathway; however, systematic investigation in endometriosis, which shares metastatic and angiogenic phenotypes, has remained unexplored.

Over the past five years, investigations have substantiated the functional significance of WNT/ β -catenin signaling in governing cellular proliferation, migration, and epithelial-mesenchymal phenotypic transitions in endometriotic cell populations^(21,22). Consequently, pathway-targeting has garnered attention as a promising intervention strategy. Given that the transcriptomic panel examined in our design encompasses WNT-associated elements, our observations demonstrate concordance with prevailing scientific understanding. Nonetheless, given the intricate nature of WNT signaling and its extensive crosstalk with parallel regulatory networks, comprehensive pathway characterization remains necessary to elucidate mechanisms.

Previous reports have documented that heparin-class compounds and structural derivatives possess the capacity to suppress neoplastic cell proliferation, directional migration, and invasive behavior, and potentially enhance

chemotherapeutic responsiveness⁽²³⁾. The pronounced attenuation of inflammation observed in the tnz7d group provides supportive evidence of previously characterized antiangiogenic and anti-inflammatory biological properties. However, definitive determination of whether tinzaparin exerts direct modulatory effects on WNT/ β -catenin signaling awaits additional mechanistic investigations.

Our experimental approach examined the relationships of leuprolide and tinzaparin with WNT/ β -catenin pathway activity, which is hypothesized to participate in the establishment of endometriosis. The results demonstrated that both pharmacological interventions modified the transcript abundance of pathway-associated genetic elements. Furthermore, findings indicated that both prophylactic and therapeutic dosing schedules produced detectable alterations in gene expression profiles. Nevertheless, subsequent investigations are necessary to ascertain whether either agent can induce lesion regression or achieve complete resolution, and to delineate optimal therapeutic dosing parameters. This work provides a preliminary characterization of prophylactic versus therapeutic applications of leuprolide and tinzaparin in a murine endometriosis paradigm, emphasizing WNT/ β -catenin pathway contributions to treatment response. Establishing molecular-level cellular effects will require expanded mechanistic studies.

In the current investigation, the effects of tinzaparin and leuprolide on experimentally induced ectopic implants were systematically evaluated in a murine model. Both compounds reduced disease severity metrics, with the delayed-tinzaparin group exhibiting significant inflammatory suppression and the corresponding leuprolide group demonstrating a notable reduction in epithelial tissue. These observations align with accumulating evidence supporting the critical involvement of WNT/ β -catenin signaling and HIF1 α -driven hypoxia/angiogenesis pathways in disease pathophysiology.

Methodological strengths of this investigation include: (1) parallel assessment of preventive and therapeutic treatment paradigms; and (2) integrated use of histopathological, immunohistochemical, and transcriptomic analyses.

Study Limitations

Recognized study limitations include: (1) Modest experimental group sizes, potentially constraining statistical power; (2) Restricted pathway coverage, with analysis limited to selected target transcripts; (3) Absence of functional cellular assays corroborating molecular observations; (4) Lack of assessment of extended treatment duration; (5) Inherent constraints on translational applicability from murine models to human disease.

This work explored alternative pharmacological approaches to the management of endometriosis, investigated disease-prevention potential, and characterized molecular pathways that contribute to lesion development. Our findings are significant for advancing the development of novel

therapeutics and expanding clinical drug indications for human patients.

Conclusion

In summary, this investigation provides preliminary evidence suggesting that tinzaparin and leuprolide exert measurable effects on endometriotic tissue. Nonetheless, comprehensive characterization of the involvement of the WNT/ β -catenin and HIF1 α pathways, coupled with validation studies in human tissue specimens and extended molecular profiling, remains essential for establishing therapeutic potential and clinical translatability.

The authors express appreciation to all contributing investigators whose efforts enabled the completion of this experimental study.

Ethics

Ethics Committee Approval: All procedures received institutional ethical endorsement from Sivas Cumhuriyet University (approval number: 307; date: 03.09.2019).

Informed Consent: Not necessary.

Footnotes

Authorship Contributions

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

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