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Does the use of low-molecular-weight heparin during pregnancy change the expression of PD-1 and PDL-1 in women with recurrent pregnancy loss?

Tekrarlayan gebelik kaybı olan kadınlarda gebelikte düşük moleküler ağırlıklı heparin kullanımı PD-1 ve PDL-1 ekspresyonunu değiştirir mi?

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Abstract

Objective: The programed cell death gene-1 ligand (PDL-1) is expressed by villous syncytiotrophoblasts, cytotrophoblasts, and fetal cells in close contact with maternal tissue and blood. Programmed cell death gene-1 (PD-1) and the PDL-1 pathway cooperate with human leucocyte antigen-G (HLA-G), expressing intermediate trophoblastic cells and syncytiotrophoblasts to inhibit the function of activated T-cells. With this mechanism, the immunosuppressive microenvironment protects the placenta. This study investigated changes in *PD-1* and *PD-L1* gene expression in patients with a history of recurrent pregnancy loss (RPL).

Materials and Methods: Sixty patients participated in the study and were divided into three groups. Group 1 (G1): healthy pregnancy, G2: RPL but not low-molecular-weight heparin (LMWH), and G3: RPL and *LMWH. PD-1* gene expression in placental tissue samples was measured by reverse-transcriptase polymerase chain reaction and PD-L1 Elisa assay, and the study was supported by histopathology.

Results: The PD-L1 value decreased significantly in G2. A significant difference was observed between the groups in *PD-1* gene expression levels in G1 and G2. It was observed that vascularization increased and the villi structures intensified in G3. In G2, there was villus dysplasia in the placenta, enlargement in the intervillous region, and fibrin deposition. It was observed that the villi structures in G3 returned to a morphology similar to that of G1.

Conclusion: T-cells are activated in patients using LMWH, and a new therapeutic strategy can be developed to prevent pregnancy loss by targeting the PD-1 pathway.

Keywords: Low molecular weight heparin, placental disorders, pregnancy complications, recurrent pregnancy loss

Öz

Amaç: Programlanmış hücre ölüm geni ligandı-1 (PDL-1), maternal doku ve anne kanı ile yakın temas halinde olan villöz sinsityotrofoblastlar, sitotrofoblastlar ve fetal hücreler tarafından eksprese edilir. Programlanmış hücre ölüm geni-1 (PD-1) ve PDL-1 yolu, etkinleştirilmiş T hücrelerinin işlevini inhibe etmek için ara trofoblastik hücreleri ve sinsityotrofoblastları eksprese eden insan lökosit antijeni-G (HLA-G) ile işbirliği yapar. Bu mekanizma ile immünosüpresif mikroçevre plasentayı korur. Bu çalışmada tekrarlayan gebelik kaybı (RPL) öyküsü olan hastalarda *PD-1* ve *PD-L1* gen ekspresyonlarındaki değişikliklerin araştırılması amaçlandı.

PRECIS: T-cells are activated in patients using LMWH, and a new treatment strategy can be developed to prevent pregnancy loss by targeting the PD-1 pathway.

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Gereç ve Yöntemler: Çalışmaya 60 hasta katıldı; üç gruba ayrıldı. Grup 1 (G1): sağlıklı gebelik, G2: RPL ama düşük moleküler ağırlıklı heparin (DMAH) kullanmıyor ve G3: RPL ve DMAH kullanıyor. Plasental doku örneklerinde PD-1 gen ekspresyonu revers-transkriptaz polimeraz zincir reaksiyonu, PD-L1 Elisa testi ile ölçüldü ve çalışma histopatoloji ile desteklendi.

Bulgular: PD-L1 değeri G2'de anlamlı olarak azaldı. G1 ve G2'deki *PD-1* gen ekspresyon düzeylerinde gruplar arasında anlamlı bir fark gözlendi. G3'te vaskülarizasyonun arttığı ve villus yapılarının yoğunlaştığı gözlendi. G2'de plasentada villus displazisi, intervillöz bölgede genişleme ve fibrin birikimi mevcuttu. G3'teki villus yapılarının G1'e benzer bir morfolojiye döndüğü gözlendi.

Sonuç: DMAH kullanan hastalarda T-hücreleri aktive olur ve PD-1 yolağını hedefleyerek gebelik kayıplarını önlemek için yeni bir terapötik strateji geliştirilebilir.

Anahtar Kelimeler: Düşük moleküler ağırlıklı heparin, plasental bozukluklar, gebelik komplikasyonları, tekrarlayan gebelik kayıpları

Introduction

Human pregnancy is considered to be a unique immunological paradigm. In contrast, immunological events during pregnancy require maternal tolerance to the semi-allogeneic fetus and maintenance of a robust immune system to protect the mother and fetus against pathogens. Pregnancy has specific cellular and molecular mechanisms that regulate and enhance the immune environment[1]. Placental development, which begins in the early pregnancy period with the invasion of fetal trophoblast cells into the decidua, is significant for the continuation of pregnancy. The survival of the developing embryo and fetus requires establishing immune tolerance by inactivating the immune system on the maternal side, with the placenta thought to be provided by the trophoblast. The trophoblastic cells in the human placenta have different types, depending on their location, specific functions, and gene expression profiles[2].

Programmed cell death gene-1 ligand (PDL-1) is expressed by villous syncytiotrophoblasts, cytotrophoblasts, and fetal cells in close contact with maternal tissue and blood[3]. Programmed cell death gene-1 (PD-1) and the PDL-1 pathway cooperate with human leucocyte antigen-G (HLA-G), expressing intermediate trophoblastic cells and syncytiotrophoblasts to inhibit the function of activated T-cells. With this mechanism, the immunosuppressive microenvironment protects the placenta[2].

Recurrent pregnancy loss (RPL) is defined as before 20 weeks of pregnancy, the loss of three or more consecutive pregnancies[4]. Approximately 1-2% of all pregnant women are affected by RPL[5]. Accepted etiologies for RPL include antiphospholipid antibody syndrome (APAS), uncontrolled diabetes mellitus, parental chromosomal abnormalities, specific uterine anatomical abnormalities, and untreated hypothyroidism. Other possible etiologies include hereditary and/or acquired thrombophilias, additional endocrine disorders, environmental causes, and immunological abnormalities. After evaluation for these etiologies, more than 33% of all cases remain unexplained RPL[6]. Low-molecular-weight heparin (LMWH) has both anticoagulant and anti-inflammatory effects. It is widely used in the treatment of recurrent pregnancy loss when used alone or in combination with other agents such as acetylsalicylic acid[7-9]. In many studies, it has been determined that LMWH shows positive results in live births and reduces complications such as preeclampsia and preterm birth[10-12]. However, the mechanisms by which LMWH may be effective during pregnancy remain unclear.

This study investigated the changes in *PD-1* and *PD-L1* gene expressions in patients with recurrent miscarriages using LMWH.

Materials and Methods

This study was reviewed and approved by the Human Research Ethics Committee (Sivas Cumhuriyet University - approval number: 2023-05/06, date: 23.05.2023). Informed consent was obtained from all participants included in the study.

This study accepted it as α=0.05, β=0.20, (1-β)=0.80. The power of the test was calculated as p=0,80248. Sixty patients participated in the study. They were divided into three groups: G1- healthy pregnancy (n=20), G2-RPL but not administered LMWH (n=20), and G3-RPL and administered LMWH (n=20). Placental tissues retrieved at birth were sampled using a random sampling method [13,14]. All patient groups were selected from singleton pregnancies and patients who had a live birth in the last trimester of their pregnancy.

Patients with APAS, uterine anomalies, genetic diseases, preeclampsia, kidney and liver disease, maternal diabetes, and intrauterine growth restriction were excluded from the study.

ELISA

Placental tissue samples taken from patients during delivery were preserved at -80 °C. The Human PD-L1 Elisa Kit (Cat. No: E3680Hu) from the Bioassay Technology Laboratory was used. The standard range of the kit was 20-7000 ng/L.

RT-PCR

Total RNA was isolated from tissues (GeneAll, Cat no:106-101). A Nanodrop spectrophotometer (Thermo Scientific, USA) was used to measure RNA concentrations. Following its catalog, HyperScript (GeneAll Cat # 601-710) was used to convert RNA samples to cDNA.

PD-1 and GAPDH (housekeeping gene) were investigated as molecular probes (qubit ssDNA Assay Kit and Life Technologies) using Sybr Green Mastermix (High Rox Dye) (Cat no: 801-051). All parameters are listed in Table 1. The 2- ΔΔCT method was used for data analysis, and the housekeeping gene was used Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Histological Analyses

Immediately after birth, the cord and placental membranes were carefully cleaned and fixed in 10% neutral buffered formalin for 24 h. Unbiased tissue sampling for each placenta was

performed using a uniform random sampling protocol[13,14]. The removed tissues were blocked by passing through increasing ethyl alcohol levels, xylol, and warm paraffin as per the routine histological tissue follow-up procedure. 3 μm sections were prepared from the blocked tissues, deparaffinized, and stained with hematoxylin-eosin dye. The morphological structures of primary, secondary, and tertiary villi, their vascular structures, and intervillous areas were evaluated in stained tissue sections. In addition, fibrin structure and syncytial trophoblast deposits on the peripheral surface of the villi were also included in the assessment[15].

Statistical Analysis

One way was the ANOVA test. All statistical analyses were performed using SPSS 22.0. Significant difference was set at $p<0.05$.

Results

PD-1 and PD-L1 ligands were studied in tissues taken from the placenta. The PD-L1 ligand was studied in tissue using the ELISA method, as shown in Figure 1.

In the study, the PD-L1 value was found in G1: 106.68±1.68 ng/mL; in G2: 93.51±2.33 ng/mL, and in G3: 103.54±0.51 ng/mL. It was observed that PD-L1 decreased significantly in G2 compared with G1 and G3 ($p<0.05$).

qRT-PCR was used to determine the expression levels of PD-1 in placental tissues, as shown in Figure 2.

When *PD-1* gene expression levels were examined, it was found to be 3.05 3.05±0.12 in G1, 1.82±0.23 in G2, and 2.82±0.84 in G3, while the control group was accepted as 1.00. There was a significant increase in the other groups compared with the PCR control group. While a significant difference was observed between the groups in G1 and G2, no significant difference was observed between G1 and G3, G2 and G3 ($p<0.05$).

After the histological evaluation in our study, an insufficient spiral artery in G2 decrease in villus volume and surface area was observed. On the other hand, in G3, the vessels are increased, and villous structures are seen in a concentrated state. There was villus dysplasia in G2 of the villi in the placenta, enlargement in the intervillous area, and fibrin deposition. An increase was also observed in the syncytial cell node located at the periphery of the villi in G2. In G3, it was observed that the villi structures returned to a morphology similar to that of the control group (Figure 3). The morphological structure of the villi on decidua was compared. Typical appearance of the villi

Table 1. Primers used

	5' Sequence 3'
PDCD1-F	ACAGTTTCCCTTCCGCTCAC
PDCD1-R	CAGTTTAGCACGAAGCTCTCC
GAPDH-F	ACGGATTTGGTCGTATTGGG
GAPDH-R	TGATTTTGGAGGGATCTCGC

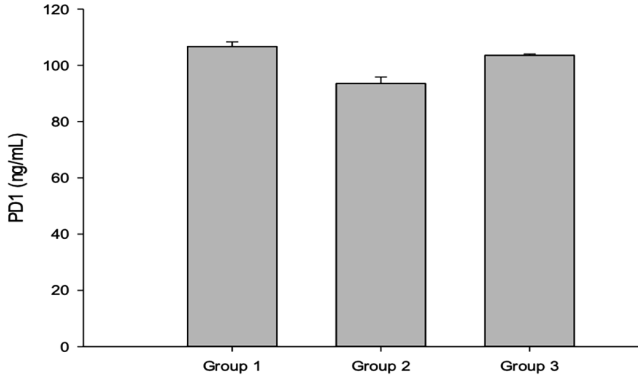

Figure 1. PDL-1 (ng/mL) value

PDL-1: Programed cell death gene-1 ligand

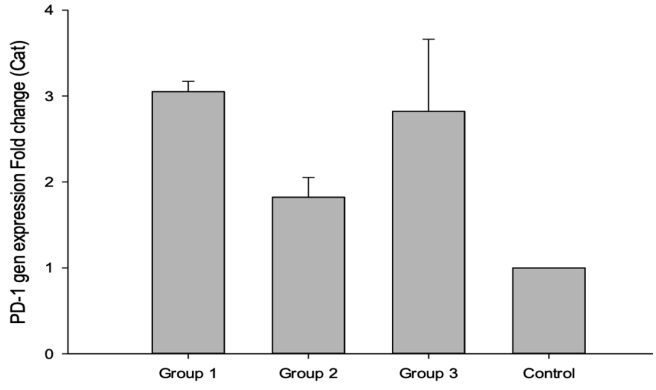

Figure 2. Change in *PD1* gene expression levels

PD1: Programmed cell death gene-1

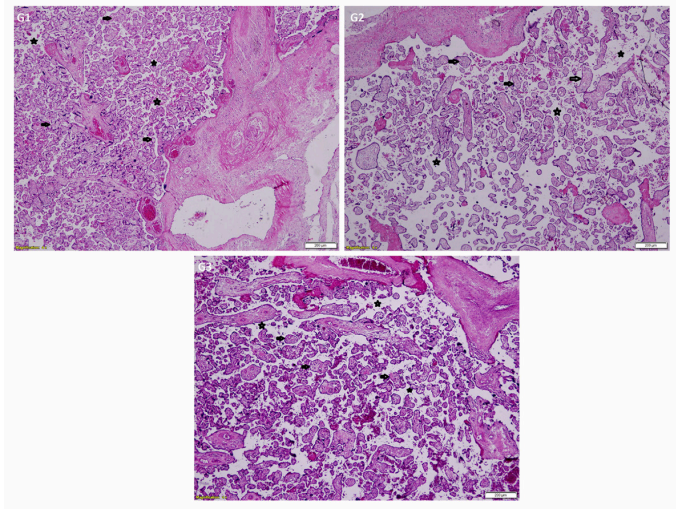

Figure 3. Comparison of the morphological structure of the villi on the decidua between the G1, G2, G3 groups of the histological changes in villous structures (arrow) and intervillous areas (star) (H&E staining, 10X Magnification)

H&E: Hematoxylin-eosin

and their arteries, villous structures with increased vascularity and blood supply, and decreased blood vessels in the separated villi were observed (Figure 4).

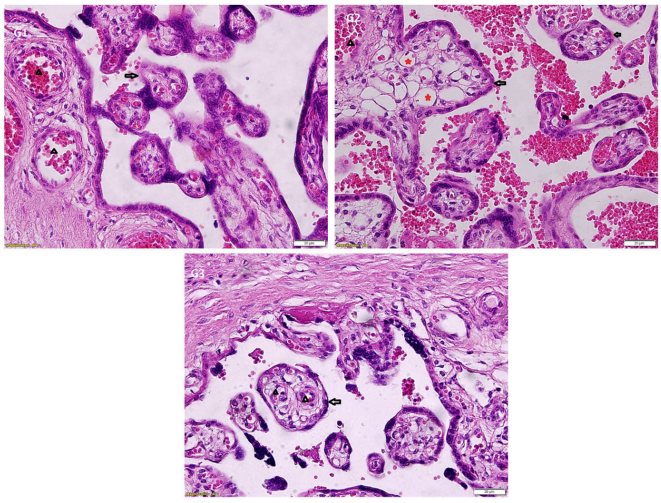

Figure 4. Comparison of structures of villi (arrow), villous arteries (triangle) and foam-like cells (red star) located in villi on the decidua between G1, G2, G3 groups (H&E staining, 40X Magnification)

H&E: Hematoxylin-eosin

Discussion

RPL is one of the most researched areas in medicine. The management of unexplained miscarriages is also challenging. Evaluation of placental morphology in the investigation of placental diseases is essential for understanding the pathogenesis of these diseases. This process involves the differentiation, migration, and division of a large number of cells and creates extensive vascularization[13].

In patients with a poor obstetric history, the area of the placental villi changes depending on the level of placental ischemia. The villous space increases with the invasion of syncytial trophoblasts, accumulation of fibrous tissue, and inadequate development of villus structures. Decreased surface area, volume, and vascularization of terminal and intermediate villi mediate maternal-fetal exchange. Perivillous fibrin deposition is characterized by the clustering of eosinophilic fibrin around the villi, and fibrin deposition impairs oxygenation around the villi, which also affects the morphology of the villi[13,15,16].

In our study, in patients with RPL who received LMWH treatment (G3), placental histopathological results showed a significant change in villi shrinkage, enlargement in the intervillous space, and fibrin deposition in the intervillous area compared with patients with RPL who did not receive LMWH (G2). However, there was no significant difference between the healthy pregnant group (G1). In the study of Ozdemir et al.,[13] it was stated that the changes in the structure of the villi and the intervillous area in the placentas of patients using LMWH were similar to our findings. However, there was no significant difference in the statistical comparison with the healthy pregnant group[13].

Deficits in maternal arterial remodeling are associated with the pathophysiology of major obstetric syndromes, including growth restriction. First, the rate at which maternal blood enters the placental intervillous space is adversely affected. Second, the involvement of the vascular smooth muscle causes a more intermittent perfusion of the placenta at the junctional site. Third, inadequate remodeling predisposes spiral arteries to acute atherotic changes. In our study, while there was foam-like structure accumulation in the spiral arteries in the decidua in the group (G2) that did not use LMWH, these structures were not observed in the healthy group (G1) and the group using LMWH (G3) (Figure 3).

Recent studies focusing on the use of LMWH have suggested insufficient evidence to apply this therapy in patients with unexplained recurrent miscarriages[17]. In Ozdemir et al.,[13] LMWH did not significantly affect the placental structure of cases with a history of recurrent miscarriage. In another study, 50 of the patients with RPL received aspirin therapy and 54 received LMWH; placental Doppler flow was similar between the groups, and the live birth rates of the groups were also similar[18].

In Rodger's meta-analysis, no effect of LMWH in reducing early pregnancy loss was observed in patients with prior placental pregnancy complications. In comparison, a statistically non-significant reduction in late pregnancy loss was observed[19]. In another study, Lu et al.[20] administered aspirin to groups of patients with miscarriage and abnormal prenatal platelet aggregation. LMWH was used in patients with high D-dimer levels, and it was observed that platelet aggregation and D-dimer levels decreased during pregnancy. An 89.2% live birth rate was reported in the group with unexplained recurrent miscarriage.

PD-1 is a molecule discovered by Freeman et al.[21] in 1992. Pdcd1 encodes PD-1 on the second chromosome. The programed cell death pathway is activated during the late stages of inflammation. In this pathway, T-cells become anergic by interacting with PD-1 on the surface of activated T-cells and its ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC) [23]. PD-1 is expressed only on activated T lymphocytes. It has also been shown that PD-1 is expressed in B lymphocytes. It is believed to have a broader spectrum than CTLA-4 in immune regulation with this feature[23]. The physiological role of PD-1 is to maintain T-cell homeostasis and balance T-cell proliferation and activation. The binding of PD-1 expressed on the surface of activated T-cells to the PD-L1 ligand generates an inhibitory signal and decreases cytokine release[24].

The PD-1/PD-L1 signaling pathway is also an adverse costimulatory pathway. PD-1 is mainly expressed on the surface of activated T-cells, whereas PD-L1 is primarily expressed on antigen-presenting cells and immunologically immune regions (such as the placenta). It has been shown that PD-L1 expression

is abundant in the placenta[25,26]. Studies have reported that PD-L1 deficiency is associated with increased fetal resorption frequency and decreased fetal survival. The interaction between PD-1 and its ligand PD-L1 plays a critical role in establishing maternal- fetal tolerance and maintaining pregnancy by regulating T-cells[27]. An important inhibitory pathway that induces T-cell anergy is PD-1/PD-L1[28]. Studies of D'addio's model of PD-L1 blockade reported that PD-L1 blockade could reduce embryo size and embryo loss in model mice while increasing Th17 and Th1 cells in peripheral lymphoid tissues[29]. However, it has been determined that the absence or decrease in PD-L1 may cause spontaneous abortion pathogenesis[26]. In our study, an increase in *PD-1* gene expression level and PD-L1 amount was observed in patients administered LMWH compared with the group that did not.

This study is essential for investigating the effects of LMWH administration, a costly and complicated treatment, on *PD-1* gene expression and placental morphology in patients with unexplained recurrent miscarriages. In conclusion, LMWH may be a promising treatment for placenta-mediated pregnancy complications, particularly for recurrent pregnancy loss, severe preeclampsia, placental abruption, and small gestational age complications. More extensive population studies are needed to better understand this issue.

Conclusion

In line with these results, it was concluded that T cells are activated in patients using LMWH, and a new therapeutic strategy can be developed to prevent pregnancy loss by targeting the PD-1 pathway.

Ethics

Ethics Committee Approval: This study was reviewed and approved by the Human Research Ethics Committee (Sivas Cumhuriyet University - approval number: 2023-05/06, date: 23.05.2023).

Informed Consent: Informed consent was obtained from all participants included in the study.

Peer-review: Internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: B.K., İ.K., Concept: B.K., C.H., Z.D.Ş.İ., Design: B.K., C.H., Data Collection or Processing: B.K., C.H., Z.D.Ş.İ., İ.K., Analysis or Interpretation: B.K., C.H., Z.D.Ş.İ., Literature Search: B.K., C.H., Z.D.Ş.İ., Writing: B.K., C.H., Z.D.Ş.İ., İ.K.

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

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