Investigation of IL-6 Expression in Placentas with Term and Preterm Premature Membrane Rupture

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Abstract

Aim: This study investigated IL-6 expression in placentas of patients diagnosed with preterm and term premature rupture of membranes (PROM) via immunohistochemical and in silico analysis.

Methods: Placentas of 40 healthy patients and 40 preterm and 40 term patients diagnosed with term PROM who gave birth in Gynecology and Obstetrics Clinic of Dicle University were subjected to histological tissue embedding protocol. IL-6 immunostaining was performed on tissues. Bioinformatic analysis was performed to explore the potential molecular mechanisms underlying role of IL-6 role in PROM.

Results: IL-6 expression was generally negative in the control group. However, it was significantly higher in both preterm and term PROM groups, with no significant difference between them. In preterm PROM group, IL-6 was recorded as positive in areas with cytotrophoblast, syncytiotrophoblast and fibrinoid accumulation, but was negative in villus connective tissue cells and vascular endothelium. In term PROM group, intense IL-6 expression was observed in trophoblastic layer, syncytial nodes and fibrinoid accumulation, but was generally negative in villous stromal and vascular endothelial cells. Enrichment analysis of IL-6-associated potential PROM targets revealed significant pathways related to microbial responses such as cellular response to lipopolysaccharide, molecule of bacterial origin and biotic stimuli, indicating that IL-6 may modulate PROM predominantly through these pathways.

Conclusions: Compared to control group, significant histopathological changes and increased IL-6 expression were observed in preterm PROM and term PROM groups, with in-silico analysis, indicating PROM increased placental inflammation and damage via IL-6.

Keywords: *Premature rupture of membrane; term; preterm; histopathology; IL-6*

1. Introduction

Amniotic fluid leakage from a defect in the amniochorionic membrane before the onset of labor at any gestational week is called premature rupture of membranes (PROM).[1] This condition is seen in 10% of all pregnancies.[2] If membrane rupture occurs before term (37th week), it is called preterm PROM and is seen in 1-2% of pregnancies. Preterm PROM is known to be the cause of more than one-third of premature births.[3,4] The latent period is the period between membrane rupture and the onset of labor. The term prolonged latent period is used when the duration of membrane rupture exceeds 24 hours. Labor begins within the following 24 hours in 85% of premature membrane ruptures at term.[3,5] Fetal membranes, amnion and chorion, are important structures that provide protection and nutrition for the embryo during pregnancy. While amnion is thin and located inside, chorion is the thick and outer layer. There is a collagen-rich connective tissue between these two layers.[6] Cells in these membranes increase with mitotic growth until the first 28 weeks of pregnancy, but they begin to regress after this process. Although amnion has more tensile strength than chorion, these membranes weaken due to biochemical and biophysical changes as pregnancy progresses.[7] One of the main reasons for this weakening is the decrease in the amount of collagen and the change in its composition.[8] These changes are the basic mechanism that leads to rupture of membranes. Towards term, i.e. when the full term of pregnancy is completed, the most common cause of rupture is physiological changes related to uterine contractions. This situation can usually be called the unnoticed

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onset of labor.[9] Intrauterine infection is an important factor in preterm (early) membrane rupture. However, in many cases, the exact cause cannot be determined.[10]

Cytokines are polypeptide molecules produced and secreted by various cells, especially immune system cells. They play important roles in many biological processes such as inflammation, cell growth, tissue healing, and the body's systemic responses to injuries [11]. Although they have hormone-like properties, they differ from hormones in some important ways.[12] In addition, recent studies have indicated that proinflammatory cytokines such as interleukin play a role in the pathogenesis of genital tract infection and inflammation in preterm labor. In light of this information, we planned to investigate the distribution of IL-6 in normal healthy placentas with premature membrane rupture.[13] In addition to IL-6, other pro-inflammatory cytokines such as TNF-α and IL-8 have also been implicated in PROM pathophysiology. TNF-α has been shown to weaken fetal membranes by inducing matrix metalloproteinase (MMP) activation, while IL-8 contributes to neutrophil recruitment and inflammatory responses in PROM cases.[14,15]

The aim of this study was to determine immunohistochemically the expression of IL-6 in term and preterm placentas with premature membrane rupture in humans and to reveal the possible role and functional relationships of this protein in these placentas.

2. Materials and Methods

2.1. A Placenta Collection

This study was conducted with the approval of the Ethics Committee of Dicle University Faculty of Medicine (date 20.12.2023 and number 2023/7). In the study, pregnant women aged between 18-49 years and without any systemic disease or secondary disease were examined at the Gynecology and Obstetrics Clinic of Dicle University Faculty of Medicine. The patient groups consisted of 40 term pregnant women who gave birth in hospital with the diagnosis of premature membrane rupture and 40 preterm pregnant women. The control group consisted of 40 pregnant women who gave birth in hospital with the diagnosis of healthy pregnancy. Placental tissue samples were taken from all three groups. The patients whose placental tissues would be collected after delivery were informed about the study and their informed consent was obtained. To minimize confounding factors, patients with systemic infections, chronic inflammatory diseases, autoimmune disorders, or maternal diabetes were excluded from the study.[16]

2.2. Placenta Tissue Preparation

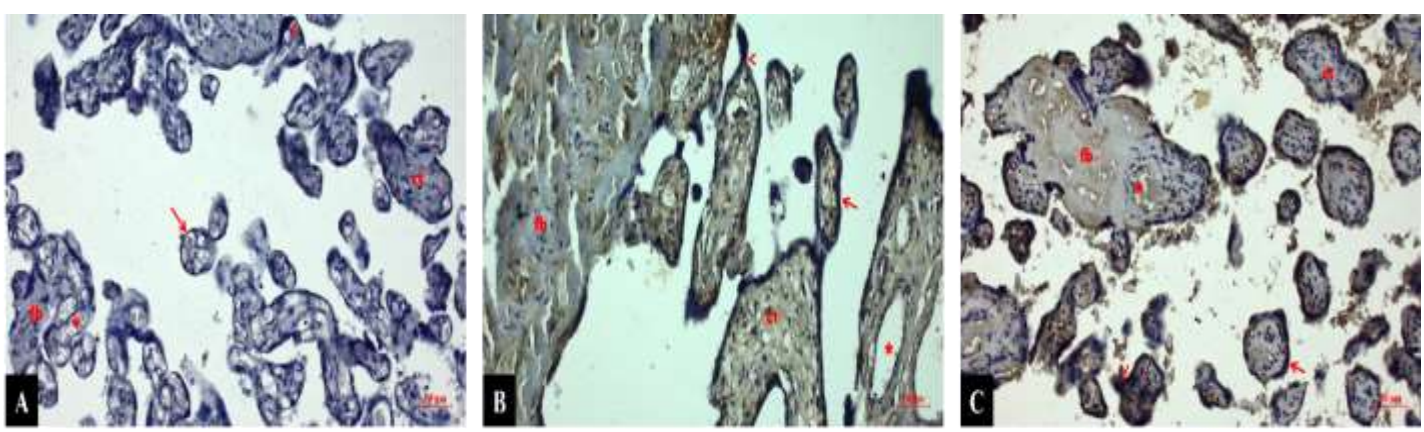
Placenta tissues taken from the maternity clinic after delivery were reduced in size in a manner suitable for histological follow-up. Placenta tissues were first kept in formalin solution for one day. Then, they were kept in running water overnight. Placenta tissues were passed through an ascending ethanol series (50%, 70%, 80%, 90%, 96% and absolute ethyl alcohol) to remove water from the tissues. Tissues were kept in xylene solution 3 times for 30 minutes to remove alcohol. Then, tissues were taken in molten paraffin liquid at 58°C. In the final stage, tissues were embedded in paraffin blocks and 4-6 µm thick sections were taken with a microtome (catalog no: Leica RM2265, Wetzlar, Germany) .

2.3. Hematoxylin-Eosin staining

Sections obtained from paraffin blocks of placental tissues were placed in a bain-marie set at 50°C. Sections were left in a 60°C oven overnight to allow the tissues on the sections to stick to the slide and to melt excess paraffin. Sections were removed from the oven, left at room temperature and allowed to cool. To remove paraffin residues from the sections, sections were kept in renewed xylene solutions for 15 minutes three times. After excess paraffin melted, sections were kept in a decreasing ethanol series (100%, 96%, 90%, 70%, 50% ethyl alcohol) for 10 minutes and excess alcohol was cleaned in distilled water. Harris hematoxylin stain was first applied to the sections for 8 minutes. Sections were kept under tap water for 5 minutes to clean excess staining. Then, the cleaned sections were kept in alcoholic (5%) eosin for 6 minutes. After the staining stage was completed, the sections were quickly dipped in a rapidly rising ethanol series. In order for the tissue in the sections to appear clear and clean, the sections were kept in xylene solutions for 3x15 minutes. Covering medium was added to the sections, they were covered with a slide and stored for examination.

Figure 1

Placental section of the control group. A) In the control group, chorionic villi maintained structural integrity, with minimal fibrinoid accumulation, Cytotrophoblast and syncytiotrophoblast layers appeared intact with negative Il-6expression; B) Preterm PROM group. Increased IL-6 expression compared to control group; C) Term PROM group. Increased IL-6 expression compared to control group. Arrow: Trophoblastic layer, arrowhead: syncytial knot, ct: connective tissue, fb: fibrinoid accumulation, *: vessels. IL-6 immunostaining, Bar: 50 µm, Magnification: 20X



2.4. Immunohistochemical Staining

Sections were washed in phosphate buffer solution (PBS) for 3x5 minutes. After epitope retrieval in ethyl diamine tetraacetic acid (EDTA) solution (pH: 8.0, catalogue no: ab93680, Abcam, Cambridge, USA), sections were treated with hydrogen peroxide solution (catalogue no: TA-015-HP, ThermoFischer, Fremont, CA, USA) for 20 minutes. Nonspecific staining was blocked with blocking solution (catalog no: TA-015-UB, ThermoFischer, Fremont, CA, USA) Primary antibody IL-6 (catalog no: sc-32296, Santa Cruz Biotechnology, Heidelberg, Germany, dilution ratio: 1/100) was dipped onto the tissues and left overnight at +4°C. following biotinylated secondary antibody (catalog no: TP-015-BN, ThermoFischer, Fremont, CA, USA), biotin-streptavidin complex was formed (catalog no: TS-015-HR, ThermoFischer, Fremont, CA, USA). Diaminobenzidine (DAB) (catalog no: TA-001-HCX, ThermoFischer, Fremont, CA, USA) was used as a chromogen. Gill III hematoxylin staining was used as a counter stain. Sections were quickly passed through an increasing ethanol series, cleared in xylene and mounted analyze and visualize with a Zeiss Imager A2 light microscope [17].

2.5. Image J Analysis

IL-6 staining intensity was measured with Image J software (version 1.53, http://imagej.nih.gov/ij). Quantification was recorded by analyzing ten fields from each preparation per group. In the samples, brown color represented positive expression of IL-6 antibody, while blue color represented negative expression of IL-6 antibody. The signal intensity (expression) from an area was calculated by dividing the intensity of IL-6 antibody by the entire area in the sample. The staining area/entire area value was calculated for each sample from ten fields. The median value was measured for the groups and analyzed for semi-quantitative immunohistochemistry scoring. Sections were imaged with a Zeiss Imager A2 light microscope. All images were processed and measured using ImageJ software [18]. For standardization, ImageJ was calibrated using predefined thresholds for brown (IL-6 positive) and blue (IL-6 negative) staining. The software was adjusted to eliminate background noise and ensure consistent measurement across all samples [19].

2.6. Bioinformatics Analysis of IL-6 and PROM Targets

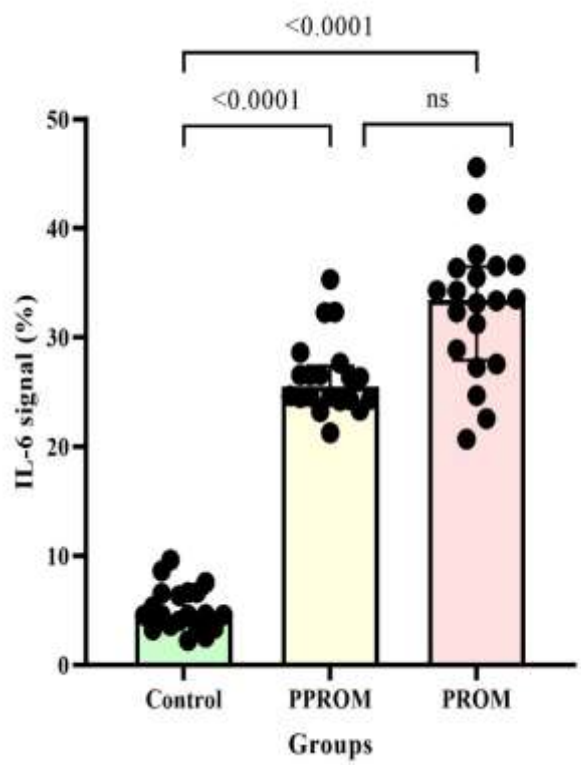
Based on the observed increase in IL-6 expression in both PROM groups, potential IL-6-associated pathways that may play a role in PROM were analyzed from a general perspective using in silico approaches. To elucidate the molecular targets associated with IL-6 in the context of PROM, we analyzed the common targets between IL-6 and the top 20 proteins potentially linked to PROM, as identified in a comprehensive proteome study [20]. The study highlighted these proteins, which exhibited significant fold changes and may be associated with PROM. These proteins are as follows: cysteine and glycine-rich protein 1 (CSRP1), hydroxyprostaglandin dehydrogenase 15-(NAD) (HPGD), pyruvate kinase M1/2 (PKM), long-chain-fatty-acid-CoA ligase 3 (ACSL3), fibulin 2 (FBLN2), high mobility group nucleosome binding domain 2 (HMGN2), HMGN3, monocyte differentiation antigen CD14 (CD14), ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 2 (BST1), scavenger receptor cysteine-rich type 1 protein M130 (CD163), lamin B1 (LMNB1), intercellular adhesion molecule 1 (ICAM1), protein tyrosine phosphatase receptor type C (PTPRC), cytochrome b-245 heavy chain (CYBB), protein S100-A9 (S100A9), Myeloperoxidase (MPO), cathepsin G (CTSG), Neutrophil elastase (ELANE), integrin subunit alpha M (ITGAM), and matrix metallopeptidase 9 (MMP9). Using Cytoscape software, a protein-protein interaction (PPI) network was constructed involving these proteins and the targets of IL-6, including 100 additional interactions (Confidence: 0.4). Subsequently, pathway intersection analysis was performed to identify potential PROM targets associated with IL-6. Finally, to investigate the role of these shared targets in molecular processes related to PROM, Gene Ontology (GO) biological process (BP) analysis was performed using ShinyGO (version 0.77, http://bioinformatics.sdstate.edu/go77/) [21]. In this platform, enrichments with an FDR value less than 0.05 were considered significant, and the top 10 Gene Ontology Biological Process (GO BP) terms were obtained based on fold enrichment [22].

2.7. Statistical Analysis

The statistical analysis of our study was performed using IBM SPSS 25.0 software (IBM, Armonk, New York, USA). Statistical data distribution in groups was evaluated with the Shapiro-Wilk test. Since the data did not conform to normal distribution, the data were presented as median (Q1-Q3). Comparisons of more than two groups were made with the Kruskal Wallis test, and pairwise group comparisons were made with the nonparametric Dunn's test. Significance was considered for p values <0.05.

Figure 2

Graphical illustration of binary comparisons of IL-6 expression between groups (Dunn's test)



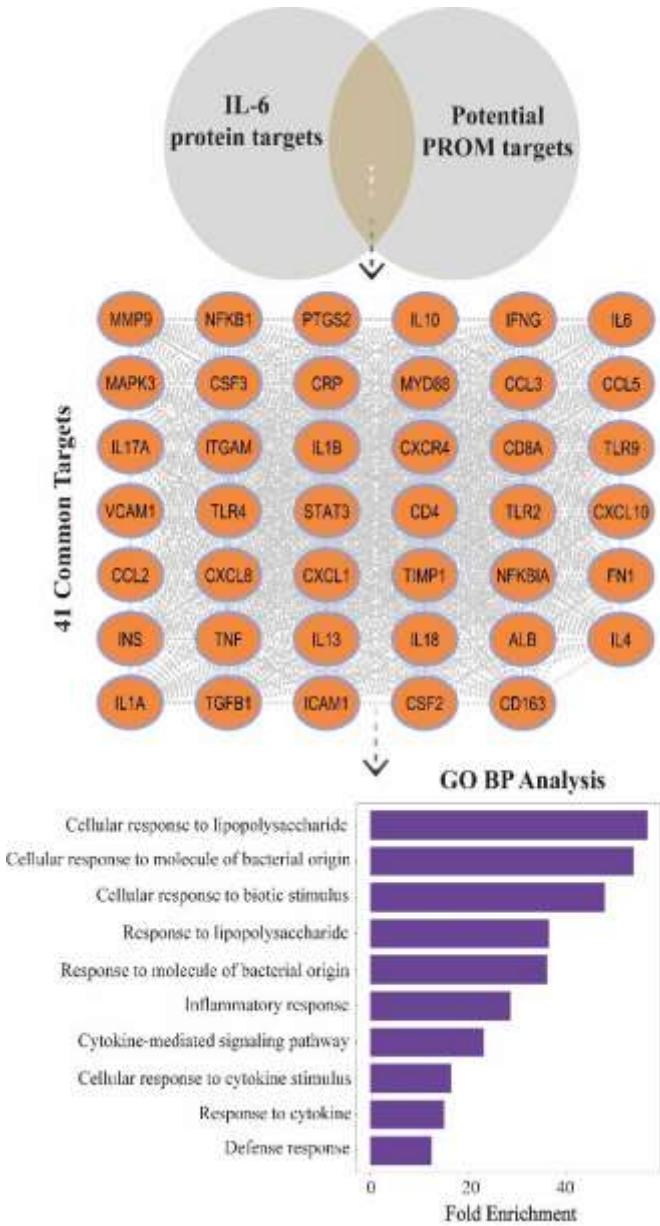
3. Results

3.1. IL-6 expression was upregulated in PROM and PPROM

Figure 1 shows IL-6 immune staining of placental sections per group. In control group, placental components were generally negative for IL-6 expression. IL-6 immune activity was not observed in the trophoblastic layer, syncytial nodes, villous stroma, vascular endothelial cells and fibronid accumulation (Figure 1A). In preterm PROM group, an increase in IL-6 expression was observed in placental tissue. IL-6 expression was recorded as positive in cytotrophoblast and syncytiotrophoblast cells, connective tissue of cells and in areas with fibrinoid accumulation. IL-6 expression was generally negative in vascular endothelium (Figure 1B). Intense IL-6 expression was generally observed in the placental components.

Figure 3

The shared protein targets of IL-6 and premature rupture of membranes (PROM) and their Gene Ontology (GO) Biological Process (BP) analysis.



TLR9: Toll-like receptor 9, ITGAM: Integrin subunit alpha M, FN1: Fibronectin 1, MAPK3: Mitogen-activated protein kinase 3, CXCR4: C-X-C chemokine receptor type 4, VCAM1: Vascular cell adhesion molecule 1, ALB: Albumin, NFKBIA: Nuclear factor kappa B inhibitor alpha, CD8A: CD8 alpha chain, INS: Insulin, TIMP1: Tissue inhibitor of metalloproteinases 1, TLR2: Toll-like receptor 2, ICAM1: Intercellular adhesion molecule 1, MYD88: Myeloid differentiation primary response 88, CCL5: C-C motif chemokine ligand 5, IL13: Interleukin 13, MMP9: Matrix metallopeptidase 9, CXCL10: C-X-C motif chemokine ligand 10, TGFB1: Transforming growth factor beta 1, PTGS2: Prostaglandin-endoperoxide synthase 2, CCL3: C-C motif chemokine ligand 3, CD163: CD163 molecule, CD4: CD4 molecule, CRP: C-reactive protein, IL18: Interleukin 18, TLR4: Toll-like receptor 4, CSF2: Colony stimulating factor 2, IL17A: Interleukin 17A, CXCL1: C-X-C motif chemokine ligand 1, IFNG: Interferon gamma, STAT3: Signal transducer and activator of transcription 3, CCL2: C-C motif chemokine ligand 2, IL4: Interleukin 4, CSF3: Colony stimulating factor 3, IL1A: Interleukin 1 alpha, CXCL8: C-X-C motif chemokine ligand 8, TNF: Tumor necrosis factor, NFKB1: Nuclear factor kappa B subunit 1, IL1B: Interleukin 1 beta, IL10: Interleukin 10, IL-6: Interleukin-6.

Intense IL-6 immune activity was observed in the trophoblastic layer (cytotrophoblast and syncytiotrophoblast), syncytial knotes and fibronid accumulation. Generally negative IL-6 expression was observed in villous stromal cells and vascular endothelial cells. Intense IL-6 expression was also observed in the immune cells of the intervillous area (Figure 1C)

3.2. PROM and PPROM showed significant increase in IL-6 expression

Semi-quantitative measurement of IL-6 immunostaining was shown in Table 1. There was a statistically significant higher IL-6 expressions in both preterm PROM and term PROM groups compared to the control group (p<0.0001).

Binary comparisons between groups were shown in Figure 2. There was no significant IL-6 expression change between PROM and PPROM groups. Both groups showed statistical differences compared to control group (p<0.0001).

Table 1

IL-6 signal intensity in groups

Signal	Control	PPROM	PROM	p value
IL-6 (Median, Q1-Q3)	4.6 (3.7-6.6)	24 (23-27)	33 (28-36)	<0.0001*

** Kruskal Wallis, PROM: Premature rupture of membrane*

3.3. PROM and IL-6 shared biological Process and targets

In the PPI networks associated with IL-6 and PROM, 41 shared proteins were identified. Gene Ontology Biological Process (GO BP) analysis of these proteins revealed several significant GO BP terms, as shown in Figure 3. These terms include: cellular response to lipopolysaccharide, cellular response to molecules of bacterial origin, cellular response to biotic stimulus, response to lipopolysaccharide, response to molecules of bacterial origin, inflammatory response, cytokine-mediated signaling pathway, cellular response to cytokine stimulus, response to cytokine, and defense response. These results indicate that IL-6 is involved in PROM by influencing cellular responses to microbial components, such as lipopolysaccharides and other bacterial molecules.

4. Discussion

Endometrial PPROM is an important obstetric complication in pregnancy and occurs in approximately 3% of all pregnancies. It is also considered to be the cause of 40% of spontaneous preterm births.[23] Preterm births account for 75% of perinatal mortality and 50% of neonatal deaths. This increases the risk of surviving newborns facing long-term health problems.[24] Therefore, PPROM and preterm births can have serious consequences affecting the health of both the mother and the newborn.[25] These data emphasize the importance of the management of PPROM and preterm births. In particular, awareness of risk factors, early diagnosis and development of treatment strategies play a critical role in reducing the negative effects of these complications. There is no definitive method for the detection of PPROM.[6,26] According to our hematoxylin-eosin findings, the placenta in the control group appears to have normal development and healthy function. The presence of fewer syncytial knots and low fibrinoid accumulation indicates that the placenta is healthy. Pathological changes observed in the placentas in the PPROM and PROM groups indicate that the

normal placental structure is disrupted. Degeneration in the chorionic villi, vascular dilatation and congestion, and hemorrhage indicate that the normal physiology of the placenta is disrupted and that there is no suitable environment for fetal development. Edema in the syncytial knots and increased fibrinoid accumulation reflect signs of tissue damage and inflammation in the placenta. Cells with a pyknotic appearance and immune cells in the interstitial area are evidence that the placenta is under inflammation and cellular stress.

Since the correct diagnosis and appropriate management of PPROM are important, various tests and methods continue to be developed.[26,27] Research on biomolecular markers is of great importance to understand the pathophysiology of PPROM, to predict this condition and to develop more effective diagnostic methods.[26] Amniochorionic membranes play an important role as the site of production of inflammatory cytokines, and these cytokines can act as inflammatory mediators leading to systemic and local changes at the fetomaternal interface.[28] Interestingly, in cases of intrauterine infection, these cytokines produced in the uterine cavity first induce a local inflammatory response. The cytokines then pass into the maternal circulation and reach the liver, inducing the release of C-reactive protein (CRP) from hepatocytes and the production of leukocytes from the bone marrow. The increase in cytokines occurs earlier than the increase in CRP and total leukocyte count.[29] IL-6 is a particularly well-known marker of infection and inflammation. IL-6 levels have been found to be significantly elevated in maternal serum, amniotic fluid and vaginal secretions of patients diagnosed with PPROM.[30] Measurement of IL-6 in maternal serum may be useful in detecting asymptomatic intrauterine infections in patients with PPROM, as it is a simpler and less invasive method.[31] A study conducted in South Nigeria showed that IL-6 concentrations in maternal serum were increased in PPROM cases compared to control cases.[32] These findings indicate that IL-6 measurement can be used as a potential early infection marker in PPROM. For these reasons, measurement of IL-6 in maternal serum is important in the early diagnosis of intrauterine infections associated with PPROM and its use in the clinic can be evaluated.[33] In our study, we examined whether IL-6 protein expression in placental tissue samples between the PPROM and control groups and whether IL-6 expression has an effect on the early diagnosis of PPROM.

In examining the underlying mechanisms of the increased IL-6 expression observed in both preterm and term PROM, our in silico analysis revealed that IL-6 significantly contributes to PROM by predominantly modulating microbial response pathways, which are consistently supported by existing literature.[34,35] For instance, IL-6's crucial role in mediating inflammatory responses to bacterial infections has been emphasized, as well as its contribution to initiating the acute-phase response in humans during such infections.[21,36] On the other hand, intraamniotic infection has been frequently correlated with PROM.[37] Consistent with our findings, it has been shown that microbial presence in the amniotic cavity contributes to increased IL-6 levels, and that IL-6, along with MMP9 and CRP, may play a key role in diagnosing intra-amniotic infections in women with PROM.[38] Although studies have reported elevated IL-6 levels in maternal serum and amniotic fluid of PROM patients, the exact mechanisms by which IL-6 contributes to PROM remain to be elucidated.[39] One study demonstrated that during inflammation, the release of IL-6 and tumor necrosis factor-alpha (TNF-α) modulates ADAMTS9, a thrombospondin motif-containing protein, which may contribute to the development of PROM.[40] These findings, combined with our bioinformatic analysis, provide valuable insights into the role of IL-6 in PROM pathogenesis, highlighting its potential as a target for future therapeutic interventions aimed at mitigating inflammation and microbial-induced membrane rupture in both preterm and term cases.

Given its strong association with inflammatory pathways, IL-6 has been proposed as a potential biomarker for PROM detection. Studies have shown that elevated IL-6 levels in maternal serum and amniotic fluid correlate with intra-amniotic infection, which is a major contributor to PROM.[41,42] Serum IL-6 measurement could serve as a minimally invasive tool for early detection and risk stratification in PROM cases, aiding in clinical decision-making

4.1. Limitations and Future Perspectives

This study provides valuable insights into IL-6 expression in term and preterm PROM; however, some limitations should be considered. The relatively small sample size may limit the generalizability of the findings, and maternal factors such as infections or systemic inflammation were not extensively analyzed. Additionally, this study focuses solely on IL-6, while other pro-inflammatory cytokines may also play significant roles in PROM pathophysiology. Future studies should include larger cohorts, consider additional biomarkers, and incorporate longitudinal data to better understand the dynamic changes in IL-6 expression throughout pregnancy.

5. Conclusion

According to our hematoxylin-eosin findings, it was determined that the placentas in the control group continued their normal development and showed healthy function. In contrast, the pathological changes observed in the placentas in the PROM and preterm PROM groups reveal that the normal placental structure was disrupted and created an unfavorable environment for fetal development. Our results emphasize the importance of the inflammatory response of the placenta in cases of premature membrane rupture and preterm birth and help us better understand the potential negative effects of these conditions on fetal development.

Our in silico analysis further supports the hypothesis that IL-6 contributes to PROM by modulating key microbial response pathways, offering a molecular framework for understanding its role in PROM pathogenesis and identifying potential targets for therapeutic intervention.

Statement of ethics

This study was conducted with the approval of the Ethics Committee of Dicle University Faculty of Medicine (date 20.12.2023 and number 2023/7).

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Conflict of interest statement

The authors declare that they have no conflict of interest.

Availability of data and materials

This study was the master thesis of Seval Özmen Ülük, Department of Histology and Embryology, Dicle University.

Author contributions

Conceptualization, F.A.; Data curation, S. Ö. Ü. and F.A.; Investigation, S.Ö. Ü. and F.A.; Methodology, F.A. and T.K.; Project administration, F.A.; Resources, F.A., A.A., F.Ş., Z.Ç. and E.A.; Software, F.A. and T.K.; Supervision, S.E. and H.A.; Validation, T.K., A.A., F.Ş. and Z.Ç.; Writing – original draft, S.Ö.Ü, F.A., T.K., A.A, F.Ş. and Z.Ç.; Writing – review & editing, F.A. and T.K.

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