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Original Research

Immunohistochemical Analysis of Vimentin and Zonula Occludens-1 in Placentas of Patients with PPROM

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Abstract

Background: We aimed to investigate the immunohistochemical staining of vimentin and zonula occludens-1 (ZO-1) expression in the placentas of pregnant women with preterm premature rupture of membranes (PPROM). **Methods**: Placentas of 25 healthy and 25 women with PPROM were fixed in 10% formaldehyde solution and further processed for paraffin wax tissue embedding. Demographic properties of patients were recorded. Placentas were histologically stained with hematoxylin-eosin and vimentin and ZO-1 expression immunostaining. **Results**: Vimentin expression was high in the decidual cells, fibroblasts, and connective tissue fibers in control group. Compared to control group, vimentin expression was decreased in the placental structures of PPROM group, where fetal membranes were degenerated and histologically irregular. Similar to vimentin expression, ZO-1 expression was also high in placental components of control group such as chorioamniotic membrane and amniotic epithelium. The PPROM group showed lower expression of ZO-1 expression in placental structures than in that of control. ZO-1 expression was significantly lowered in regions where fetal membrane integrity was weakened and lost. **Conclusions**: We suggest that ZO-1 and vimentin expression may show alteration in etiology premature rupture of membrane.

Keywords: PROM; amniotic membrane; junction; histopathology

1. Introduction

Premature rupture of the membrane (PROM) is defined as rupture of fetal membranes at any time before labor occurs. If this happens before the 37th week of pregnancy, it is called preterm premature rupture of the membrane (PPROM). Although the etiology of PROM and PPROM is not known exactly, membrane weakening, intra-amniotic infection, uterine ovarian distension, cervical shortening, and vaginal bleeding are risk factors. The incidence of PPROM varies between 3–5% in all pregnancies. The diagnosis of PROM is made based on the patient's history, physical examination, laboratory, and ultrasound (USG) examination. As a treatment, delivery is recommended for those over 34 weeks [1–3]. The principal treatment for PPROM is close hospitalization and monitoring of mother and fetus. antibiotic therapy also common in PPROM patients due to risk of infection. In addition to antibiotic therapy, corticosteroid therapy is also acceptable if delivery is imminent. For some cases where pulmonary system is insufficient, tocolytic medication could be administered. Also, close monitoring maternal and fetal well-being is important such as USG examination, measurement of amniotic fluid levels, fetal heart rat detection of chorioamnionitis, *etc*. [4].

The molecular mechanism of PPROM is not fully known; however, it is known that the structural integrity of the chorioamniotic membrane is impaired. Extracellular matrix (ECM) is a tissue-specific non-cellular component that contains many structural and functional proteins in its structure. These proteins in the ECM provide stability, and the dynamic change here may cause various pathologies (such as abnormal placentation, PPROM, uterine rupture, cervical insufficiency) [5]. Matrix metalloproteinase (MMP)-9 concentration was found to be higher in patients with PPROM compared to patients with preterm delivery and membrane intact [6]. Pan *et al*. [7] studied proteomic profiling in the placentas of pregnant patients with preterm delivery. In the study, it was noted that proteolysis has a role in fetal membrane rupture and the ECM is affected by proteolysis [7].

Vimentin is a type III intermediate filament and is widely found in the cytoplasm. It is responsible for the stabilization and cell plasticity of intracellular components. It has a role in various biological events such as wound healing, fibrosis, and metastasis [8,9]. Mary *et al*. [10] found that that vimentin expression was significantly higher in preeclamptic placentas than in normotensive placentas. The

authors concluded that vimentin is associated with apoptosis during placentation and needed to be frutherly analyzed [10]. Zonula occludens-1 (ZO-1) is a tight junction protein that acts as a scaffold between actin and cytoskeleton and transmembrane proteins. ZO-1 connects neighboring epithelial cells and provides cellular integrity [11,12]. Pidoux *et al*. [13] studied human placenta and found that ZO-1 was involved in trophoblastic differentiation and regulated the human placental development.

This study aimed to investigate role of the vimentin and ZO-1 in placentas of patients with PPROM in order to elucidate alterations in cellular structure and integrity in placentas of PPROM.

2. Methods

2.1 Patients

Ethical approval was taken from Dicle University Medical School, Non-interventional Clinical Trials Ethical Committee (2020/68). In our study, 25 healthy women and 25 women with PPROM were included. Healthy women were considered as control group which had spontaneous labour at full term with intact membranes. Similar to PROM, PPROM criteria were included leakage of fluid, vaginal discharge, vaginal bleeding, and pelvic pressure without contractions. Patients with any secondary systemic disease (diabetes, chronic kidney problems, cardiac problems) were excluded. Placentas were obtained from Department of Gynecology and Obstetrics. Demographics and laboratory parameters were recorded for each patient. All patients were approved and agreed to participated in. The patient informed consent form was read to all patients, and they signed the forms.

2.2 Histological Tissue Processing

Histological sampling was done from placentas of control and PPROM. Dissected placental samples were further analyzed for histological evaluation. Samples were fixed in 10% formaldehyde (Merck, Darmstadt, Germany) and dehydrated through grading alcohol series and incubated in paraffin wax. 5 µm sections were cut from paraffin blocks and stained for hematoxylin eosin dye and immune staining [14].

2.3 Immunohistochemical Examination

Placentas of patients with PPROM were immunestained for the vimentin and ZO-1 antibodies. PPROM is mainly thought be a cause of fetal membrane dis-integrity and rupture study for This study was designed to investigate these antibodies because they are involved in cell integrity, cellular junctions, and cytoskeleton.

Placental sections were dewaxed, hydrated in grading alcohol series, and washed in distilled water. Epitope retrieval was inducted by ethyl diamine tetra acetic acid (EDTA, (Merck, Darmstadt, Germany) solution (pH = 8.0) for 15 minutes in a microwave oven at 700 W. 3% hydrogen peroxide (H_2O_2, Thermo Fischer, Fremont, CA, USA) was dropped on slides to block endogen peroxidase activity. After washing in phosphate buffered saline (PBS), sections were incubated with vimentin and ZO-1 (AFG Scientific, Northbrook, IL, USA, 1/100) overnight at +4 °C. Sections were biotinylated and allowed to react with streptavidin peroxidase solution (Thermo Fischer, Waltham, MA, USA) for 15 minutes. After PBS washing, diaminobenzidine (DAB, Thermo Fischer, Fremont, CA, USA) chromogen was used as a chromogen to observe color change. The reactions were stopped with PBS solution and sections were counter stained with hematoxylin dye (Merck, Darmstadt, Germany). Slides were analyzed under a light microscope [15].

2.4 Statistical Analysis

Statistical analysis was done using the IBM SPSS 25.0 software (IBM, Armonk, NY, USA). The data were recorded as median (Q1–Q3). Data distribution was done by Shapiro-Wilk test. Binary group comparisons were done with Mann Whitney U test.

3. Results

3.1 Patients' Characteristics in Study Groups

Clinical parameters of patients were listed in Table 1.

3.2 Vimentin Findings in Control and PPROM Groups

Immune activities of vimentin and ZO-1 were shown in Fig. 1. Intense vimentin expression was observed in membranes of decidual cells in the control group. In addition, positive Vimentin expression was observed in fibroblasts and collagen fibers from connective tissue cells. Negative vimentin expression was detected in vascular endothelium and syncytial nodes. Vimentin expression was higher in extracellular matrix (Fig. 1a). In the PPROM group, vimentin expression was mild positive in the amniotic epithelial membrane, decidual cells and some blood vessels. Vimentin expression decreased in the degenerated placental structures (Fig. 1b).

3.3 ZO-1 Findings in Control and PPROM Groups

Intense ZO-1 expression was detected in the chorioamniotic membrane of the placental sections in the control group. Intense ZO-1 immune activity was also observed in the amniotic epithelium. Relatively weak ZO-1 expression was observed in the vascular endothelial wall and decidual cells (Fig. 1c). In the placental sections of the PPROM group, membrane integrity was lost, and disintegrated, and negative ZO-1 expression was detected in these areas. Similarly, ZO-1 expression was found to be negative in amniotic epithelial nuclei and vessel walls. It was observed that ZO-1 expression decreased in areas where the amniotic membrane was degenerated (Fig. 1d).

Immunohistochemical staining of placental sections were scored as 0: none, 1: slight, 2: moderate, 3: in-

Table 1. Clinical properties of patients.

Parameters	Control	PPROM
Maternal age, year	27.35 ± 2.59	31.28 ± 5.57
Gravida, n	1.36 ± 0.85	4.93 ± 1.58
Parity, n	1.13 ± 0.92	2.73 ± 1.84
Gestational age at delivery (week)	38.76 ± 1.56	32.43 ± 2.05
Gestational age at PPROM (week)	35.62 ± 2.25	36.05 ± 2.58
Birth weight, g	3583.29 ± 287.28	2012.30 ± 329.83
BMI, kg/m^2	27.48 ± 4.28	29.35 ± 5.20

PPROM, preterm premature rupture of membranes; BMI, body mass index.

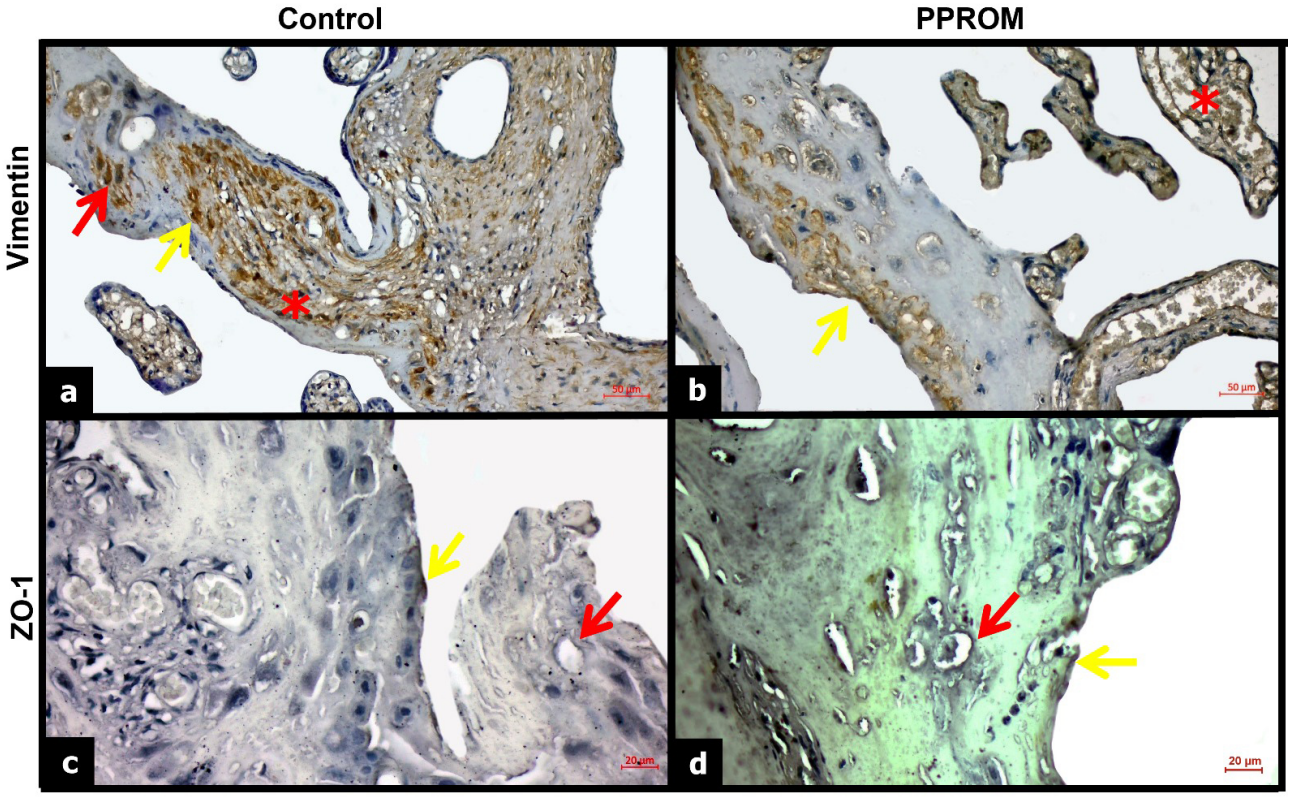

Fig. 1. Immune staining of placentas of control and PPROM groups. (a) Positive vimentin expression in decidual cells (red arrow), fibroblast cells (yellow arrow) and collagen fibers (red star), Scale Bar: 50 μm, magnification: 20×. (b) Positive vimentin expression in amniotic epithelial membrane (red arrow), decidual cells (yellow arrow), blood vessels (red arrowhead), Scale Bar: 50 μm, magnification: 20×. (c) Positive ZO-1 expression in the chorioamniotic membrane (red arrow), vessel endothelial wall (yellow arrow), Scale Bar: 50 μm, magnification: 40×. (d) Positive ZO-1 expression (yellow arrow) in the membrane (red arrow) and endothelium of the vessel walls, Scale Bar: 50 μm, magnification: 40×. ZO-1, zonula occludens-1.

tense. Placental sections were analyzed by two blind expert pathologists. Results were shown in Table 2 and Fig. 2.

4. Discussion

The amniotic membrane or amnion is the innermost layer of the fetal placenta and is characterized by a thick basement membrane and an avascular stroma. The amniotic membrane completely surrounds the embryo and defines the boundary of the amniotic cavity containing the amniotic fluid. The amniotic membrane has pluripotent cells and is currently used in tissue transplantation [16]. Extracellular matrix (ECM) is unique to each tissue and has various biochemical and biophysical properties. Molecules such as collagens, proteoglycans, laminin, and fibronectin are associated with the ECM and form the tissue-specific ECM [17]. Zhu *et al.* [18] studied PROM and control to investigate NOD1 expression in placenta and fetal membranes. They found that NOD1 expression was higher in PROM placentas and fetal membranes compared to control placentas, the authors stated that NOD1 regulated the immune response and with NF-κB, it was involved in development of PROM [18].

Table 2. Statistical analysis of immune staining by groups.

Expression	Control	PPROM	Significance
Vimentin, median (Q1–Q3)	3.0 (3.0–3.0)	1.0 (0.0–1.5)	$p < 0.0001$
ZO-1, median (Q1–Q3)	3.0 (2.0–3.0)	0.0 (0.0–1.0)	$p < 0.0001$

In this study, the expression levels of vimentin, one of the intermediate filaments, and ZO-1 protein belonging to the occluding protein family, which plays a role in the cytoskeleton, were investigated in patients with premature rupture of the membrane (PPROM). Özgökçe *et al.* [19] recorded high levels of vimentin expression in the placentas of patients with HELLP (Hemolysis, Elevated Liver enzymes and Low Platelets) syndrome and suggested that this was due to endothelial dysfunction. Similarly, Sak *et al.* [20] reported that high vimentin levels in the placentas of patients with HELLP syndrome may cause placental dysfunction. Janzen *et al.* [21] investigated the levels of matrix metalloproteinases (MMPs) in patients with preterm PROM (PPROM). The authors stated that the epithelial-mesenchymal transition (EMT) of amniotic cells affect birth and that EMT weakens the tensile strength of the amnion [21]. Lai *et al.* [22] studied 30 cases of PROM and examined their placentas in terms of tensile strength. The authors found that type IV collagen, vimentin and desmin in PROM group were dysplasia and aged in fetal membranes [22]. Another study showed that epithelial cells shed from fetal membranes of PROM indicated higher expression of vimentin protein [23]. The result of this study showed that intense vimentin expression was observed in the membranes of decidual cells, connective tissue fibers and cells. In the PPROM group, vimentin expression was decreased compared to control group. The expression was observed in the amniotic epithelial membrane, decidual cells and blood vessels. Accordingly, with literature, results confirm that vimentin expression was significantly decreased in the placental structure where cellular junctions were abundant (Fig. 1a,b and Fig. 2).

Fetal membranes (amnion and chorion membranes) are composed of collagen-rich ECM. The ECM is a complex composed of fibrous proteins, providing structural support to fetal membranes, and communicating with the cytoskeleton [24]. Marzioni *et al.* [25] stated that downregulation or dysregulation of zonula occludens-1 (ZO-1) expression in the placentas of patients with hydatidiform mole affects the epithelial transformation of chorionic villi. In a study investigating patients with GDM, they stated that occluding expression in the placenta was downregulated, and this change may increase the susceptibility to inflammatory events [26]. Li *et al.* [27] stated that ZO-1 level decreased in the fetal membranes of patients with PROM who had intrauterine infection, inflammation downregulated ZO-1 expression, and this could cause PROM. The result of this study showed that chorioamniotic membrane and amniotic epithelium showed high expression of ZO-1

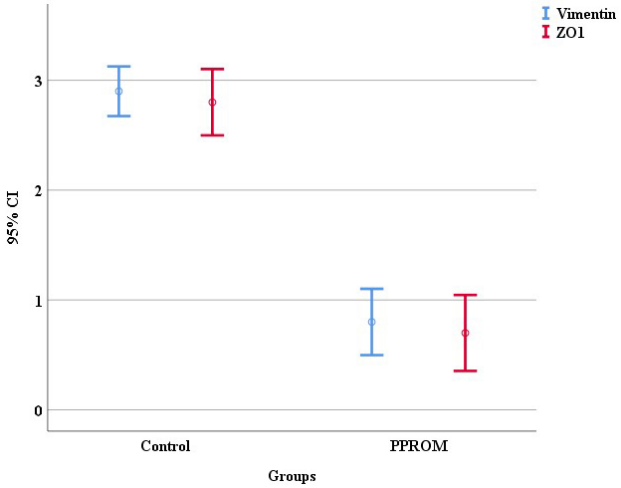

Fig. 2. Illustration of error bars of immune activity vimentin and ZO-1 in control and PPROM groups. 95% CI, 95% confidence interval.

in control group. In the PPROM group, ZO-1 expression was decreased in fetal membranes (Fig. 1c,d and Fig. 2). Consistent with studies, result showed that ZO-1 expression was significantly lowered in regions where fetal membrane integrity was weakened and lost. Li *et al.* [27] investigated the expression of ZO-1 in fetal membrane of PROM placentas. The authors revealed that significantly reduction of ZO-1 expression in fetal membrane, chorion and amnion epithelium and it was negatively correlated with intrauterine infection [27].

Della Rosa *et al.* [28] studied risk of preterm birth by considering a multitude of intrauterine and extrauterine factors. The study contributes a holistic risk assessment methodology for preterm birth in clinical practice, offering a nuanced understanding of risk factors and individualized profiling. The selected factors effectively estimated the degree of preterm birth risk, providing valuable insights for personalized care [28].

Vimentin and ZO-1 is essential for epithelial cells and involved in tensile strength and cytoskeleton of amnion epithelial cells. These proteins could be used for PPROM patients with different biological samples to elucidate pathogenesis of PPROM.

5. Conclusions

The hypothesis that the cause of preterm membrane rupture is the weakening of the chorioamniotic membrane, and the deterioration of its structural and functional integrity is emphasized. We suggest that deterioration of the

cell-cell junction complex of chorioamnionic membrane in early membrane rupture and distribution of vimentin in the cytoskeleton during inflammation may be indicated by ZO-1 and vimentin expression respectively. More studied are needed to elucidate the etiology of preterm premature rupture of membrane.

Availability of Data and Materials

All data points generated or analyzed during this study are included in this article and there are no further underlying data necessary to reproduce the results.

Author Contributions

All the authors have contributed to the document retrieval, conception, and design of this study. Material preparation, data collection and analysis, and patient follow-up were conducted by FZ, FA, ISE, MCT, ED and CO. Histopathological follow-up was performed by FA, MCT and ED. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, ethical approval was taken from Dicle University Medical School, Non-interventional Clinical Trials Ethical Committee (approval number: 2020/68).

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Conflict of Interest

The authors declare no conflict of interest. Süleyman Cemil Oğlak is serving as one of the Guest editors of this journal. We declare that Süleyman Cemil Oğlak had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Paolo Ivo Cavoretto.

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