The Investigation of Caspase-3 and Tumor Necrosis Factor-Alpha Expression in Placentas of Patients with Preterm Premature Rupture of Membranes

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Abstract

Background: Caspase-3 is involved in the execution of apoptosis and is widely used as an apoptotic marker. Tumor necrosis factor-α (TNF-α) released from activated macrophages has various functions such as modulation of cell growth and differentiation, immunoregulation, coagulation, and regulation of endothelial cell function. This study investigated the immunohistochemical staining of caspase-3 and TNF-α 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 processed for routine histological tissue processing. Placentas were stained with hematoxylin-eosin, caspase-3, and TNF-α immunostaining.

Results: Normal placental histology was observed in the control group. Amniotic epithelium, vascular structures, and fibrinoid accumulation were histologically normal. Leukocyte infiltration, thinned vessel walls with dilatation and congestion, syncytiotrophoblasts, and fibrinoid accumulation were increased in the PPROM group. The immune activity of caspase-3 expression was mainly negative in placental components such as syncytiotrophoblasts, vascular endothelium, fibrinoid accumulation, and macrophages in the control group. In the PPROM group, caspase-3 positive reaction was increased in the amniotic membrane and epithelium, endothelial cells, fibrinoid accumulation, and areas of inflammatory cell infiltration. In the control group, negative TNF-α expression was observed in the placental membranes and structures. In the PPROM group, TNF-α expression was increased in inflammatory cells, endothelial cells, and syncytiotrophoblasts. Conclusions: Placentas of patients with PPROM showed loss and weakened membranes with increased placental pathology, and increased expression of caspase-3 and TNF-α. We suggest that caspase-3 and TNF-α signaling pathways can be used as a marker in the progression of PPROM.

Keywords: caspase-3; tumor necrosis factor-alpha; placenta; premature rupture of membranes

1. Introduction

Preterm premature rupture of membranes (PPROM) is defined as the fetal membrane rupture before labor and 37 weeks of gestation. This complication is the identifiable leading cause of preterm birth and has been one of the main causes of infant morbidity and mortality [1–3]. Recently, term or preterm rupture of fetal membranes occurs not only as a result of the tensile and shear forces of uterine contractions, but also as the result of a weakening process of membranes. It has been reported that acute inflammation, in which proteolytic enzymes and activating cytokines are released, is effective in the subgroup of preterm infants with PPROM [4]. 26–50% of placentas born following PPROM have the characteristics of acute chorioamnionitis [5]. Romero et al. [6] reported that intraamniotic inflammation occurs in 37% of preterm birth cases before 37 weeks of gestation.

The healing mechanisms of damaged tissue are divided into four overlapping stages: (i) hemostasis, (ii) inflammation, (iii) migration and proliferation, and (iv) resolution and remodeling. Unlike adult tissues, fetal tissue is much simpler to heal [7]. Inflammation is minimized, fetal tissue is usually not vascularized, and granulation tissue is not formed. It provides rapid healing of fetal wound healing without leaving any trace [8]. Macrophages play an important role in the healing of fetal tissues. Circulating monocytes migrate to sites of injury where they differentiate into tissue macrophages, and tissue-resident macrophages are also involved in wound healing [9]. It has been reported that increased oxidative stress in women with premature rupture of membranes predisposes them to rupture by accelerating early cellular senescence, age-related inflammation, and proteolysis. One of the factors involved in the endogenous and exogenous mechanisms associated with the increased risk of PPROM is the occurrence of programmed cell death or apoptosis [10,11].

Caspases are proteases that degrade functional and structural proteins in the cell, leading to programmed cell death. They are synthesized as zymogens and converted
to proteases. Caspase-3 is involved in the execution of apoptosis and is widely used as an apoptotic marker [12]. Tumor necrosis factor-α (TNF-α) released from activated macrophages has various functions, such as modulation of cell growth and differentiation, immunoregulation, coagulation, and regulation of endothelial cell function [13].

There is limited study on the PPROM from a histopathological perspective. Considering the risk factors of PPROM, this study aimed to investigate the molecular level signaling and histological examination of apoptotic and inflammation processes (caspase-3 and TNF-α), which may be effective in the change and signaling of membrane structure in cases with membrane rupture.

2. Materials and Methods

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. All placenta samples were obtained by the Department of Obstetrics with the help of the physician. All patients were informed about the data and experimental protocol. For each patient, biochemical and clinical parameters were noted after their approval. All patients read and signed informed consent form.

A total of 50 patients aged 20–40 years with a singleton pregnancy who were admitted to the Department of Obstetrics were recruited, including 25 cases of PPROM patients (gestational week <37 weeks), and 25 cases of healthy pregnancies as control (gestational week ≥37 weeks) group. Gestational age was calculated according to the first-trimester ultrasound scan for all pregnancies.

The diagnosis of PPROM was made using a speculum examination by visualizing the amniotic fluid pooling in the posterior vaginal vault or amniotic fluid leakage from the cervix, together with a positive test for the presence of Placental Alpha Microglobulin-1 (Anmasure®) in vaginal fluid[14]. The exclusion criteria of all pregnancies in the present study were: structural or chromosomal abnormalities of the fetus, being at the active stage of labor, previable rupture of membranes (<24 weeks of gestation), multiple pregnancies, intrauterine fetal death, signs of fetal hypoxia, signs of intrauterine growth restriction (IUGR), vaginal bleeding, or any medical complication such as diabetes mellitus, gestational hypertensive disorders, thyroid disease, or known malignancy.

2.1 Histological Tissue Processing

Placenta specimens were obtained from the chorion frondosum of the maternal surface of the placenta during the cesarean delivery. Fetal membrane specimens were acquired near the fetal membrane rupture. Placental samples were maintained in sterile conditions until the experiment, and deidentified by a physician. Samples were directly soaked into 10% formaldehyde solution and dissected for histological dye experiments. The placental tissues were later dehydrated in increasing alcohol series, soaked in xylol solution, and embedded in paraffin blocks. 4 µm sections were cut and stained for hematoxylin-eosin staining[15].

2.2 Immunohistochemical Examination

Placental sections were cleared in xylol solution, dehydrated in alcohol, and cleared in distilled water. Epitope retrieval was induced by ethylenediamine tetra-acetic acid (EDTA) solution (pH = 8.0) for 15 minutes in a microwave oven at 90 °C. After the sections were cooled down, they were rinsed in phosphate-buffered saline (PBS) three times for 5 minutes. 3% hydrogen peroxide (H2O2, catalog no: TA-015-HR, Thermo Fischer, Fremont, CA, USA) was dropped onto slides to block endogenous peroxidase activity. After washing in PBS, sections were incubated with rabbit polyclonal caspase-3 and TNF-α (catalog no: A60144 and A42662, AFG Scientific, Northbrook, IL, USA, 1/100) overnight at 4 °C. Sections were dipped into PBS, and biotinylated antibody solution (catalog no: TP-015-BN, Thermo Fischer, Fremont, CA, USA) was dropped onto slides for 14 minutes. Sections were reacted with streptavidin peroxidase solution (catalog no: TS-015-HR, Thermo Fischer, Fremont, CA, USA) for 15 minutes. After PBS washing, diaminobenzidine (DAB, catalog no: TA-001-HCX, ThermoFischer, Fremont, CA, USA) chromogen was used to observe color change for a maximum of 10 minutes. The reactions were stopped with PBS solution and sections were stained with hematoxylin dye. Slides were analyzed under a light microscope [16].

2.3 Statistics

Statistical analysis was done using the IBM SPSS 25.0 software (IBM, Armonk, New York, USA). The data were recorded as mean ± standard deviation (SD) or median (minimum-maximum). Data distribution was done by the Shapiro-Wilk test. Binary group comparisons were done with the Mann-Whitney U test or the independent t-test. Hypotheses were two-sided, and a p-value < 0.05 was considered statistically significant.

Each section was analyzed with Image J software (version 1.53, LOCI, University of Wisconsin, Madison, WI, USA). The staining intensity (expression) of caspase-3 and TNF-α for each group was recorded. Statistical analysis was applied to analyze the mean difference between groups in terms of expression (signal intensity).

3. Results

We analyzed the demographic variables, clinical characteristics, and pregnancy outcomes between PPROM and control groups in Table 1. We found no significant differences between the groups regarding maternal age, gravidity, parity, body mass index, and the mean value of laboratory parameters, including hemoglobin, white blood cell, and C-reactive protein.

Histopathological and immunohistochemical staining of control and PPROM placentas were shown in Fig. 1.
In the control group (Fig. 1a), the epithelium was slightly cuboidal at the location of the amniotic membrane and slightly protruding towards the lumen. The basement membrane was regular, the capillaries in the lower part were smooth and the endothelial cells were flat. In some areas in the lower part of the chorionic membrane, vascular structures, solitary dispersed inflammatory cell infiltrates, and prominent macrophage cells were seen. Few fibrinoid accumulations were observed. Syncytial nodes and bridges and blood vessels were observed to be regular (Fig. 1a). Inflammatory cell infiltrates were seen at the border of the chorioamniotic membrane in the PPROM group (Fig. 1b). Thinning of the vessel wall, thinning, and ruptures of the amniotic membrane, which is one of the typical features of premature rupture of membranes, were observed. Enlargement of the syncytial nodes, dilatation of the thinned vessels, congestion, and thrombosis were observed. It was observed that fibrinoid accumulation increased and vacuolar structures were prominent. Tears and increased cell infiltration were observed around the vessel (Fig. 1b).

In the control group, it was observed that the amniotic epithelium was regular, and caspase-3 expression was negative. Negative expression was also observed in syncytial nodes, vascular endothelium, and fibrinoid accumulation. Caspase-3 expression was also negative in Hoffbauer cells, especially in some areas where macrophage activity was evident (Fig. 1c). In the PPROM group, when the chorioamniotic membrane was examined, a caspase-3 positive reaction was observed especially in the interrupted amniotic membrane and in the dysfunctional amniotic epithelium. Thinning was observed in adjacent areas of the chorioamniotic membrane. Caspase-3 positive reaction was observed in some endothelial cells. Positive caspase-3 expression was detected especially in thinned areas, around the vessel wall, in fibrinoid accumulation, and in areas of inflammatory cell infiltration. However, caspase-3 showed negative expression in areas with syncytial nodes and syncytial bridges (Fig. 1d).

In the control group, negative TNF-α expression was observed in the areas of the membrane, especially in the epithelium. TNF-α expression was positive, especially in endothelial cells of small vessels in the area extending from the amniotic membrane to the chorionic membrane. Negative expression was detected in the membranes and amniotic epithelium. Macrophage cells were seen from place to place in the area where the chorionic membrane was located. We think that macrophage activity in the control group developed due to cytokine activity against possible inflammation (Fig. 1e). In the PPROM group, inflammatory cell infiltration was observed in the lower part of the basement membrane and TNF-α expression was significantly increased in some macrophages. TNF-α expression was observed in endothelial cells in dilated and thrombosed vessels. It was observed that syncytialization was weak in terms of inflammation in syncytial nodes and syncytial bridges, and TNF-α expression was negative. It can be said that thinning occurs due to the deterioration of the basement membrane and the increase in inflammation in the endothelium and amniotic membrane (Fig. 1f).

Staining intensity was done by Image J® software version 1.53. Hematoxylin and DAB chromogen staining intensity for c-3 and TNF-α expression of all placentas were analyzed. Fig. 2 shows the immune activity of Caspase-3 and TNF-α in groups by percentage (%). Both expression intensity was increased in PPROM placentas compared to control placentas (p < 0.05).

4. Discussion

In this study, we found that the placentas of patients with PPROM showed numerous pathologies such as loss of fetal membrane integrity, inflammation, thinned vascular walls, and elevated fibrinoid accumulation. The caspase-3 and TNF-α expressions were also increased relative to the placentas of control patients.

Of the fetal membranes, the inner and thinner one is the amnion, and the outer and thicker one is the chorion. Between the amnion and the chorion, there is a connective

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Table 1. The analysis of demographic variables, clinical characteristics, and pregnancy outcomes between PPROM and control groups.

<table>
<thead>
<tr>
<th></th>
<th>PPROM group (n = 25)</th>
<th>Control group (n = 25)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age, years</td>
<td>28 (21–38)</td>
<td>29 (22–39)</td>
<td>0.613</td>
</tr>
<tr>
<td>Gravidity</td>
<td>3 (1–6)</td>
<td>3 (1–5)</td>
<td>0.514</td>
</tr>
<tr>
<td>Parity</td>
<td>2 (0–5)</td>
<td>2 (0–4)</td>
<td>0.143</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>27.5 (19.4–32.7)</td>
<td>27.1 (19.8 ± 33.8)</td>
<td>0.877</td>
</tr>
<tr>
<td>Hemoglobin, g/dL</td>
<td>10.88 ± 0.73</td>
<td>11.26 ± 0.92</td>
<td>0.209</td>
</tr>
<tr>
<td>White blood cell, /mm³</td>
<td>13.57 ± 3.97</td>
<td>12.59 ± 3.71</td>
<td>0.760</td>
</tr>
<tr>
<td>C-reactive protein, mg/L</td>
<td>8.3 (0.9–61.2)</td>
<td>7.14 (0.8–36.2)</td>
<td>0.419</td>
</tr>
<tr>
<td>Latency period, days</td>
<td>16.46 ± 13.1</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Birth week</td>
<td>31.88 ± 3.68</td>
<td>38.36 ± 1.19</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Birth weight, g</td>
<td>1982.6 ± 407.4</td>
<td>3268.47 ± 261.8</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

PPROM, Preterm premature rupture of membranes; N/A, Not available.
Fig. 1. Hematoxylin eosin staining (a,b) and immunostaining (c–f) of the placental sections. Bar: 50 µm, magnification: 20×. (a) Control group: Regular cubical epithelium (black arrow), regular basement membrane (red arrow), chorionic vacuolar structures (black star). (b) PPROM group: Inflammatory cell infiltration (black arrow), vessel wall thinning (green arrow), enlargement of syncytial nodes (red arrow), dilatation, congestion, and thrombosis (black star), fibrinoid accumulation (blue star). (c) Control group: Negative caspase-3 expression in amniotic epithelium (black arrow), negative caspase-3 expression in syncytial nodes (red arrow), vascular endothelium (arrowhead), hofbauer cell (green arrow), and fibrinoid accumulation (black star). (d) PPROM group: Positive caspase-3 expression in the amniotic epithelium (black arrow), chorioamniotic membrane thinning (red arrow), vessel wall (arrowhead), fibrinoid accumulation (blue star), and inflammatory cells (black star). (e) Control group: Tumor necrosis factor-α (TNF-α) positive in endothelial cells (black star), macrophage cells (black arrow). (f) PPROM group: Positive TNF-α expression in inflammatory cells (black arrow) and macrophages (black star), dilated and thrombosed vessels (red arrow).

tissue rich in collagen. Amnion has more tensile strength than chorion. As pregnancy progresses, the weakening of the membranes occurs due to biochemical and biophysical changes. The amount of collagen decreases. The main mechanism in the rupture of membranes is the decrease in the amount of collagen in the structure of the membranes and the change in its composition [17,18].

It has been reported that it may occur as a result of different factors, including physiological rupture of fetal membranes, mechanical modifications, remodeling of extracellular matrix components, cell degenerations, and apoptosis of amniotic cells. Since the amnion has poor vascularization, it has been shown that it cannot be applied for blood agglutination and epithelial wound healing in coagulation of the skin, and for reclosing the damaged amnion [19,20]. It has been observed that the weakening of the extracellular matrix in the fetal membrane due to collagen degradation makes pregnant women active for PPROM. This process is thought to be the result of membrane stretching, which directly leads to tissue degeneration, as well as biochemical remodeling, inflammation, and apoptosis [21,22]. In our study, the control group showed normal placental histology. Amniotic epithelium, vascular structures, and fibrinoid accumulation were regularly observed. Compared to the control group, dense inflammatory cell infiltration, thinned vessel walls with dilatation and congestion, increased syncytial nodes, and fibrinoid accumulation were observed in the PPROM group.
Caspase-3 is the main member of the caspase family involved in apoptosis. Before apoptosis begins, caspase-3 is activated, and almost all apoptosis proceeds by this signaling pathway. Immunohistochemical examination of the amniotic membrane in PPROM cases shows caspase-3 expression in amniotic epithelial cells and chorionic cytotrophoblast cells, while its expression is limited in mesenchymal and reticular cells of the matrix. This indicates that apoptosis occurs in the amnionic and chorionic layers. As previously described by Negara et al. [23], caspase-3 expression, which indicates apoptosis, was 9.75 times higher in amniotic epithelial cells in PPROM cases. In Harirah et al. [24] study on the role of apoptosis in the amniotic membranes of patients who delivered at term and patients who underwent cesarean section (artificial rupture), showed that the apoptotic index was three times higher in the region of spontaneous rupture in the chorionic trophoblast, with higher expression of active caspase-3. In our study, the control group showed mainly negative caspase-3 expression in placental components such as syncytial nodes, vascular endothelium, fibrinoid accumulation, and macrophages. In the PPROM group, caspase-3 positive reaction was increased in the amniotic membrane and epithelium, endothelial cells, fibrinoid accumulation, and areas of inflammatory cell infiltration. Moreover, we also observed that caspase-3 expression was higher in the placentas of patients with PPROM than in that of the control group.

PPROM induces choriodecidual inflammation. Bacterial invasion from the choriodecidual space stimulates the release of cytokines such as TNF-α and interleukin-1 (IL-1) from the fetal membrane and decidua. Kucukgul et al. [25] showed that both maternal and cord plasma TNF-α levels were significantly higher in PPROM cases. In their study, Li et al. [26] showed that TNF-α increased in the placental tissues of women with premature rupture of membranes. Accordingly, in our study, the control group presented negative TNF-α expression in the placental membranes and structures. In the PPROM group, TNF-α expression was increased in inflammatory cells, endothelial cells, and syncytial nodes. Similar to caspase-3 expression, we found that TNF-α was statistically more expressed in the placentas of patients with PPROM than in that of the control group.

Weakening of the extracellular matrix in the fetal membrane due to collagen degradation makes pregnant women active in PPROM. This process appears to be a result of biochemical remodeling, inflammation, and apoptosis as well as membrane stretching, which directly leads to tissue degeneration.

5. Conclusions

Herein, we investigated the histopathology of the amniotic membrane and the role of inflammatory cytokine TNF-α with caspase-3 expression. In the present study, increased TNF-α expression induced the angiogenic process and lead to an apoptotic pathway, via elevated caspase-3 expression. High inflammation with cell death deteriorates the amniotic membrane integrity, causing promoting the progression of PPROM. During placental development, pregnancy complications can cause histological alterations in placental structures, such as amniotic membrane,
and placental villi. PPROM may be caused by membrane weakening, infections, excess uterine distension, and vaginal bleeding. We suggest that the caspase-3 and TNF-α signaling pathways may be an important marker in the progression of PPROM.

Availability of Data and Materials
Data is available on request from the authors.

Author Contributions
ED, FA, SCO, and GEA designed the research study. ISE, FA, GEA, and ÖK performed the research and experimental protocols. FA and SCO analyzed the data and statistics. ISE, ED and FA wrote the manuscript. FA and SCO made the critical review of the manuscript. All authors contributed to editorial changes in the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate
Ethical approval was taken from Dicle University Medical School, Non-interventional Clinical Trials Ethical Committee (2020/68). All patients read and signed informed consent form.

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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 Michael H. Dahan.

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