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The Relationship between the Expression of Beclin-1, Caspase-3, and Apoptosis-Inducing Factor Amniotic Membrane in the Premature Rupture of Membranes

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ABSTRACT

Premature rupture of membranes, known as PROM, constitutes a pathological condition that significantly contributes to maternal and neonatal morbidity and mortality. Despite its prevalence, the underlying molecular mechanisms of PROM, particularly those involving apoptosis and autophagy in the amniotic membrane, remain incompletely understood. Therefore, this study is aimed at analysing the association between expression of Beclin-1, Caspase-3, and AIF (Apoptosis-Inducing Factor) in the amniotic membrane and the incidence of PROM. A cross-sectional study was conducted involving 62 term parturients, divided into 2 groups (PROM and non-PROM). Amniotic membrane samples were collected post-delivery, and expression levels of Beclin-1, Caspase-3, and AIF were assessed using immunohistochemistry. Data analysis was executed using the Mann–Whitney U test. The findings demonstrated that the mean expression level of Beclin-1 was lower in the PROM group than in the non-PROM group, but the difference was not statistically significant ($p = 0.083$). Caspase-3 and AIF expression levels were significantly higher in the PROM group compared to the non-PROM group ($p < 0.001$), indicating the involvement of apoptotic pathways in the pathogenesis of PROM. These results support the critical role of apoptosis in the rupture of the amniotic membrane and may serve as a foundation for the development of diagnostic and preventive strategies.

Keywords: Premature Rupture of Membranes, Beclin-1, Caspase-3, Apoptosis-Inducing Factor, Apoptosis, Autophagy

Introduction

Premature rupture of membranes, known as PROM, constitutes a pathological state characterised by the rupture of the amniotic membrane prior to the commencement of labour. In addition, it represents a key factor in neonatal and maternal mortality and morbidity, leading to significant focus within the field of perinatology.[1-3] PROM may occur at either term or preterm gestations, requiring prompt medical intervention to prevent serious clinical consequences for the mother and the fetus alike. Globally, the condition has been reported in roughly 5 - 10% of pregnancies, where 80% of cases are observed at term[4] and 3 - 8% at preterm.[5] In Indonesia, PROM prevalence ranges from 4.5% to 7.6%, with a report from Sanglah General Hospital in Denpasar showing a notably higher rate of 14.62%, predominantly occurring at term.[6] According to previous studies, PROM has long-term adverse effects on both maternal and neonatal health.[7] In mothers, it increases the risk of intrauterine infections, such as chorioamnionitis.[8] PROM can lead to severe complications in neonates, including sepsis, pneumonia, RDS (respiratory distress syndrome), necrotising enterocolitis, neonatal sepsis, as well as intraventricular haemorrhage.[9,10]

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Preterm PROM accounts for 30 - 40% of preterm births and significantly contributes to neonatal morbidity.[11] Despite its multifactorial aetiology, the pathophysiological mechanisms underlying the condition remain incompletely understood. Several contributing factors include infection, hormonal imbalances, nutritional deficiencies, behavioural risks (smoking, coitus), and mechanical causes (multiple pregnancy, polyhydramnios, macrosomia). The latest findings demonstrate that collagen degradation in the extracellular matrix, together with programmed cell death via autophagy and apoptosis in the amnion-chorion layers, also plays a critical role in membrane rupture.[12-14]

Apoptosis and autophagy are vital cellular mechanisms in preserving homeostasis, including within the amniotic membrane.[15] Several studies have shown that autophagy is responsible for recycling damaged cellular components triggered by stressors, such as infection, hypoxia, or nutrient deprivation.[16,17] Impaired autophagic activity in fetal membranes has been associated with preterm labour and PROM, and can be evaluated through expression of autophagy-related proteins, including Atg16L1, Atg12, Atg7, Atg5, Atg3, and Beclin-1.[18]

Apoptosis is critically involved in the pathogenesis of PROM. Caspase-3, a principal effector of apoptosis, has been found to be upregulated in cases of preterm PROM.[19] This apoptotic process mainly proceeds through the intrinsic, mitochondria-mediated pathway controlled by the Bcl-2 protein family.[20] In addition to the caspase-dependent route, a caspase-independent pathway is also involved, typically activated by DNA damage due to infection and oxidative stress, and mediated by the release of pro-apoptotic proteins, including endonuclease G and AIF (apoptosis-inducing factor).[21,22] Given the multifaceted regulatory pathways associated with the pathogenesis of PROM, assessment of these molecular markers can provide crucial insights for diagnostic and therapeutic strategies.

Considering the importance of clarifying the underlying molecular pathways of PROM, particularly the roles of autophagy and apoptosis in maintaining amniotic membrane integrity, further studies are

essential to develop effective preventive and therapeutic approaches aimed at reducing maternal and neonatal complications. The limitations of performing invasive interventions during pregnancy pose significant challenges for molecular studies in this context. Therefore, this study is aimed at analysing the role of apoptotic and autophagic processes in the amniotic membrane in relation to the incidence of PROM. To address this objective, immunohistochemistry was used to evaluate expression levels of Beclin-1, Caspase-3, and AIF in post-delivery amniotic membrane samples. This method allows for precise localization and quantification of protein expression within tissue architecture, enabling a comparative analysis between PROM and non-PROM groups. The integration of this method with clinical-pathological correlation is expected to enhance insight into the molecular pathways implicated in PROM and to inform the development of targeted bimolecular diagnostic and preventive strategies.

Materials and Methods

Ethical considerations

This study included the collection of amniotic membranes from postpartum women with or without PROM. Participants were provided with information regarding the study and were required to provide written informed consent prior to participation. The confidentiality of participant identities was maintained, and consent was obtained before any disclosure of identifying information. This study was approved by the Ethics Committee of the Faculty of Medicine, Universitas Andalas, Padang, West Sumatra (No: 586/UN.16.2/KEP-FK/2023).

Study population and sample collection

This cross-sectional study was executed over a period of 2.5 years, with sample collection performed at the Department of Obstetrics and Gynaecology of RSUD Bangkinang and RSIA Husada Banda Bangkinang, Riau, Indonesia. The study population comprised term pregnant women who delivered with or without PROM. The inclusion criteria included term gestational age, live foetus, and voluntary participation confirmed through a written informed consent. The exclusion criteria included cases with polyhydramnios, multiple pregnancies, macrosomia, infection during pregnancy (leukocyte count >16,000 cells/μL), and pregnancy complications such as diabetes mellitus, hypertension, and antepartum hemorrhage. The total sample size consisted of 62 participants, divided evenly into PROM (n = 31) and non-PROM (n = 31) groups.

Participant management and screening

Participants were administered a questionnaire and an informed consent form to confirm eligibility according to the predefined inclusion criteria. Screening involved collection of demographic and clinical data, such as gestational diagnosis, age, education level, occupation, parity history, and other relevant personal information.

Collection of amniotic membrane samples

Amniotic membranes were collected from participants who met the inclusion criteria by providing a signed consent letter from the patient and immediately after delivery by providing a signed consent letter. The membranes were collected longitudinally from the site of rupture, approximately 2 cm around the edge of the tear. Subsequently, the specimens were rinsed with 0.9% NaCl solution and fixed in 10% buffered formalin before being submitted to the laboratory for further analysis.

Histopathological examination of the amniotic membrane

Amniotic membrane samples were prepared at the Department of Anatomical Pathology, RSUD Bangkinang, followed by immunohistochemical staining at the Department of Anatomical Pathology, Faculty of Medicine, Universitas Andalas. Initially, paraffin blocks were cut into 4 μm sections using a microtome, and affixed to poly-L-lysine-coated slides. Sections were deparaffinised in xylene, and subsequently subjected to graded rehydration in 100%, 96%, and 70% ethanol, then washed in distilled water, each lasting 5 minutes. Epitope was obtained using the HIER (Heat Induced Epitope Retrieval) technique in citrate buffer (pH 6.0) and microwave treatment over 10 minutes. Sections were rinsed 3 times with PBS (Phosphate buffered saline) (pH 7.4), each lasting 5 minutes. Endogenous peroxidase activity was quenched by incubation in 3% H_2O_2 in PBS over 3 minutes and 0.3% H_2O_2 in PBS over 30 minutes, and subsequently subjected to 3 additional PBS washes. Non-specific binding was suppressed using 2% NGS (Normal Goat Serum) in PBS over 20 minutes at ambient temperature. Slides were subjected to overnight incubation at 4°C using primary antibodies in a humid chamber. The primary antibodies employed comprised anti-Beclin-1 (dilution 1:100), anti-AIF (1:200), and anti-Caspase-3 (1:200). After incubation, slides were rinsed with PBS (pH 7.4) 3 times, each wash lasting 5 minutes. Thereafter, sections were incubated with biotinylated Goat Anti-Rabbit IgG (H+L), BA-1000-1.5 (1:100), over a 30-minute period at room temperature, followed by 3 PBS washes. An avidin-biotin complex was employed over a 30-minute period under room temperature conditions. Antigen visualisation was achieved using 3,3'-Diaminobenzidine (DAB) chromogen in Tris-HCl buffer (pH 7.6), followed by 3 washes in distilled water. Counterstaining was performed with haematoxylin, and slides were rinsed with distilled water for 10 minutes. Dehydration was carried out in graded ethanol (70%, 96%, and 100%), each for 5 minutes, and immersed in xylene two times, each lasting 5 minutes. Finally, coverslips were mounted with Entellan mounting medium.

Data analysis

Data analysis was executed using both bivariate and univariate approaches. Univariate analysis aimed to present the features of every study variable, typically providing frequency distributions and percentages. Bivariate analysis was used to evaluate statistical relationships between independent and dependent variables. To assess the association between expression levels of Beclin-1, AIF, and Caspase-3 with the incidence of PROM, the SPSS (Statistical Package for the Social Sciences) version 20 was used. As a parametric test required normally distributed data, the Kolmogorov-Smirnov test was applied to assess data normality. Results showed that expression data for Beclin-1, Caspase-3, and AIF were not normally distributed. Despite data transformation, normality assumptions remained unmet; hence, the Mann–Whitney U test, functioning as a non-parametric equivalent, was used.

Results and Discussion

Respondent demongraphic characteristics

In this study, respondent characteristics were evaluated using the frequency distribution of several general variables known to influence the development of PROM, including age, educational background, occupation, and parity type (Table 1). The majority of respondents in both the PROM and non-PROM groups were aged between 20 and 35 years (90.3%), which corresponded to the reproductive age and is physiologically favourable for a normal pregnancy. As such, age within this range did not directly increase the risk of PROM. However, membrane integrity could be compromised by other underlying conditions, as seen in extreme age groups (<20 or >35 years), who were more vulnerable to amniotic membrane disruption due to tissue immaturity or degeneration.[22,23]

In terms of educational attainment, the most common highest level of education completed by respondents in both the non-PROM (16/51.6%) and PROM (20/64.5%) groups was senior high school. Education served as an essential determinant in maternal health, as it strongly correlated with health literacy. Limited education could lead to a lack of understanding of pregnancy danger signs, hygiene during gestation, and adherence to antenatal care (ANC), all of which are crucial in preventing PROM.[24,25]

Most respondents were housewives, as observed in both the non-PROM (23/74.2%) and PROM (22/70.9%) groups. Housewife is a status that generally provided greater opportunity for self-care during pregnancy. However, factors such as excessive domestic workload, household stress, sociodemographic challenges, and limited access to healthcare could indirectly contribute to PROM. Inadequate social and economic support remained the most prominent contributing factor.[7,26] With respect to parity type, the non-PROM group showed a higher proportion of multiparous women (20/64.5%), while in the PROM group, the distribution was relatively even between primiparous and multiparous women (15/48.4%). This result was consistent with prior evidence that

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showed that primiparous women were at greater risk of PROM due to cervical rigidity, suboptimal uterine biomechanical adaptation, and lack of experience in recognising and managing abnormal pregnancy symptoms, thereby increasing the likelihood of premature membrane rupture.[27]

Table 1: Frequency Distribution of Respondent Characteristics

Characteristic	PROM (%)	Non-PROM (%)
Age:		
<20 years	1 (3.2)	-
20-35 years	28 (90.3)	28 (90.3)
>35 years	2 (6.5)	3 (9.7)
Education:		
Elementary School (SD)	2 (6.5)	-
Junior High School (SMP)	2 (6.5)	4 (12.9)
Senior High School (SMA)	20 (64.5)	16 (51.6)
University (PT)	7 (22.5)	11 (35.5)
Occupation:		
Housewife (IRT)	22 (70.9)	23 (74.2)
Civil Servant	6 (19.4)	5 (16.1)
Public Officer	2 (6.5)	-
Self-employed/Trader	1 (3.2)	3 (9.8)
Parity:		
Primipara (Para 1)	15 (48.4)	10 (32.3)
Multipara (Para 2-4)	15 (48.4)	20 (64.5)
Grande Multipara (Para >4)	1 (3.2)	1 (3.2)

Expression of Beclin-1, Caspase-3, and AIF in amniotic membrane

The mean Beclin-1 expression level was lower in the PROM group as opposed to the non-PROM group, though the difference was not statistically significant (Figure 1). Beclin-1 is instrumental in autophagy initiation via complex formation with VPS34 (Class III PI3K), which facilitates autophagosome formation, essential for recycling damaged cellular components triggered by oxidative stress and inflammation, which are common features of pathological pregnancies.[28,29] The observed reduction in Beclin-1 expression among PROM cases suggested altered autophagy pathways, rendering amniotic membrane cells more vulnerable to oxidative damage. This could occur through alternative autophagy routes, accumulation of misfolded proteins and organelles, and increased apoptosis, including the release of proteolytic enzymes like matrix metalloproteinases (notably MMP-9).[30] Moreover, reduced Beclin-1 levels allow for enhanced interaction with Bcl-2, promoting both alternative autophagy and apoptotic signalling, thereby weakening membrane integrity. Despite the lack of statistical significance, the trend indicated that decreased Beclin-1 regulated key biological signalling pathways, contributing to the pathogenesis of PROM.[12] These results emphasized the need for further investigations into Beclin-1 regulatory interactions with MMPs, oxidative stress, and inflammatory markers to better establish a causal relationship between autophagy dysfunction and PROM incidence.

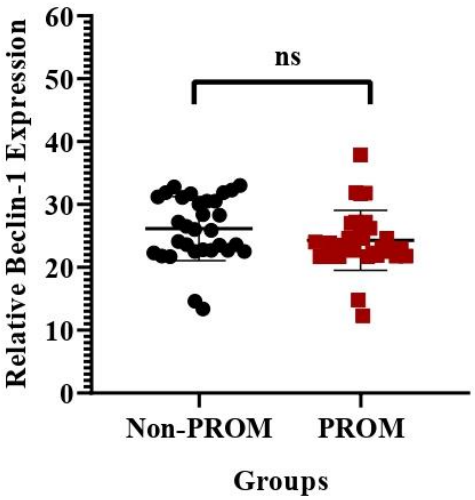

Figure 1: Mean Expression of Beclin-1 in the Non-PROM and PROM Groups
Beclin-1 expression was measured using immunohistochemistry. The results showed a decrease in Beclin-1 expression in the PROM group, with an average expression score of 24.29 and an area percentage of 4.76%. In comparison, the non-PROM group exhibited an average expression score of 26.16 and an area percentage of 5.08%. 'ns' indicates no statistically significant difference.

In addition, the reduced Beclin-1 expression reflected disruption in the canonical autophagy pathway, where Beclin-1 typically formed an initiation complex with Vps34, ATG14L, and AMBRA-1.[31,32] However, even under pathological conditions where Beclin-1 expression was downregulated, autophagy still proceeded through compensatory non-canonical pathways. In this adaptive response, the loss of canonical autophagy triggered negative feedback and metabolic stress sensors that activate alternative autophagic signalling.[33,34] One such compensatory pathway was the Rab9-dependent autophagy route, which operated independently of ATG5, ATG7, and Beclin-1.[35,36] Research has shown that when canonical autophagy is inhibited due to suppressed expression of core autophagy components, autophagosomes still formed through vesicular fusion from the trans-Golgi and late endosomes with lysosomes, mediated by Rab9.[37] Activation of this pathway was more pronounced as Beclin-1 expression declined, suggesting a compensatory shift toward Rab9-mediated autophagosome formation, with reduced maturation efficiency.[37]

Beyond Rab9, reduced Beclin-1 stimulated compensatory transcriptional activation via Transcription Factor EB (TFEB).[38] Under conditions of impaired autophagy, TFEB translocated to the nucleus and promoted the transcription of autophagy- and lysosome-associated genes, including ATG9, LAMP1, and Rab7/9.[39] Consequently, Beclin-1 downregulation functioned as an upstream signal for TFEB activation and the induction of non-canonical pathways. This also activated energy and oxidative stress sensors like AMPK or AMP-activated protein kinase as well as FOXO3, both of which mediate autophagy independently of Beclin-1.[39,40] AMPK directly phosphorylated ULK1, initiating autophagy without Beclin-1, while FOXO3 promoted expression of ATG12, ATG4, and LC3.[41,42] Beclin-1 downregulation therefore signals basal autophagy failure, which in turn activated AMPK and FOXO3 as part of a survival mechanism to maintain minimal autophagic capacity.

In this molecular context, Beclin-1 downregulation not only impeded canonical autophagy but also actively initiated alternative compensatory signalling through Rab9 activation, TFEB nuclear translocation, and AMPK–FOXO3 stimulation. However, these alternative pathways were generally slower and less efficient than the canonical route. Consequently, in pathological conditions such as PROM, these compensatory mechanisms failed to counterbalance the

effects of oxidative stress, inflammation, and amniotic membrane disintegration. This suggested that Beclin-1 downregulation was not merely a biomarker, but a critical regulatory node marking the transition from protective autophagy to tissue failure.

A significant ($p < 0.001$) increase in mean Caspase-3 expression was observed in the PROM group (38- 42) compared to the non-PROM group (27.69) (Figure 2). Caspase-3 is a critical protease in apoptosis, executing programmed cell death by cleaving several intracellular substrates.[43] In PROM, elevated Caspase-3 suggested excessive apoptosis in amniotic membrane tissues, which compromised membrane strength.[44] This process was primarily driven by the intrinsic apoptosis pathway, initiated by oxidative stress, infection, or acute inflammation that disrupted mitochondrial membrane integrity, resulting in cytochrome release as well as subsequent Caspase-9 and Caspase-3 activation.[43] Caspase-3 then mediated the degradation of structural matrix proteins like type I and type III collagen and intercellular adhesion molecules, resulting in diminished membrane cohesion and elasticity. Interestingly, despite higher mean expression, the percentage of Caspase-3-positive areas within the PROM group was lower (6.92%) as opposed to the non-PROM group (8.36%), suggesting that Caspase-3 expression in PROM could be more localized to structurally critical regions rather than diffusely distributed. Collectively, these results supported the concept that apoptosis dysregulation, as evidenced by increased Caspase-3 expression, played a direct role in weakening membrane integrity and elevating PROM risk.

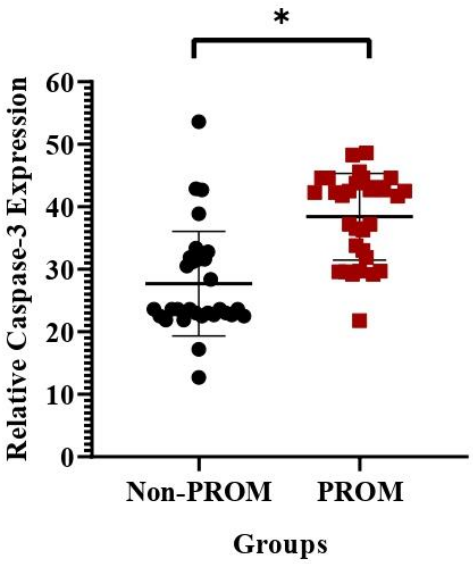

Figure 2: Mean Expression of Caspase-3 in the Non-PROM and PROM Groups
Caspase-3 expression was measured using immunohistochemistry. The results showed an increase expression of Caspase-3 in the PROM group, with an average expression score of 38.42 and an area percentage of 6.92%. The non-PROM group exhibited an average expression score of 27.69 and an area percentage of 8.36%. '*' indicates statistically significant difference at $p < 0.001$.

Assessment of Apoptosis-Inducing Factor (AIF) expression showed a significant increase in the PROM group ($p < 0.001$), with an average expression score of 39.93 and an area percentage of 9.71% (Figure 3). In comparison, the non-PROM group showed an average AIF expression score of 27.93 and an area percentage of 9.54%. This indicates the critical role of AIF in PROM pathogenesis through caspase-independent apoptosis as has been demonstrated in previous study.[43] AIF is a mitochondrial flavoprotein that translocated to the nucleus upon severe oxidative stress or cellular injury, leading to chromatin condensation and DNA fragmentation.[45] In PROM, elevated oxidative stress due to intrauterine infection, inflammation, or mechanical overstretching of the fetal membranes could drive AIF release,[46] thereby accelerating apoptosis in amniochorionic membrane cells and reducing membrane strength. Moreover, heightened AIF expression reflects local antioxidant defence failure, thereby exacerbating tissue damage and impairment of regenerative potential.[43,47,48]

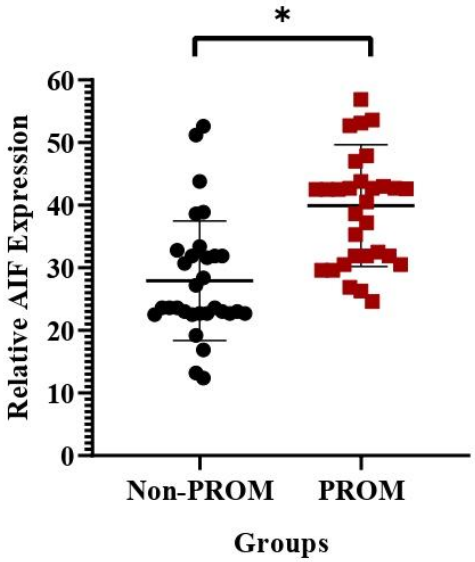

Figure 3: Mean Expression of AIF in the Non-PROM and PROM Groups. The results showed an increase expression of AIF in the PROM group, with an average expression score of 39.93 and an area percentage of 9.71%. The non-PROM group showed an average expression score of 27.93 and an area percentage of 9.54%. '*' indicates statistically significant difference at $p < 0.001$.

Conclusion

The results of this study highlight a robust link between PROM and apoptotic mechanisms in the fetal membranes. Expression levels of Caspase-3 and AIF were significantly elevated within the PROM group compared to the non-PROM group, indicating the activation of both caspase-independent and caspase-dependent apoptotic pathways in PROM pathogenesis. However, Beclin-1, an essential autophagy regulator, showed lower expression in the PROM group although not significantly different from that of the non-PROM group. These findings emphasize that apoptosis dysregulation plays a more prominent role than autophagy dysfunction in compromising the structural integrity of the fetal membranes, thereby contributing to PROM onset. This study provides a foundation for the formulation of therapeutic, diagnostic, and preventive measures aimed at reducing PROM incidence through biomolecular approaches.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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