

# Differences in the Expression of Apoptosis inducing Factor in Amniotic Cells in Premature Rupture of Membranes and Without Premature Rupture of Membranes in Preterm Labor

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**Abstract:** ***Background:** Preterm premature rupture of membranes (PROM) is one of the important causes of preterm birth associated with perinatal morbidity and mortality and high maternal morbidity. The pathogenesis of preterm PROM is multifactorial where apoptosis initiated by apoptosis inducing factor (AIF) in mitochondria of amniotic cells is thought to play an important role. This protein is the first protein to initiate the caspase-independent apoptosis mechanism through nicotinamide adenosine dinucleotide hydrogen (NADH) oxidase in the mitochondria. The purpose of this study was to prove the differences in AIF expression of amniotic cells in PROM cases and without PROM in preterm labor. **Method:** We conducted this cross-sectional study in the ER Maternity Room at Sanglah Hospital Denpasar for 6 months from July 2019 to December 2019. The samples were preterm pregnant women who were willing to participate in the study using simple consecutive random sampling. The material is the amniotic membrane amniotic tissue on the side of the tear after delivery and examination of AIF expression using immunohistochemical techniques at the Integrated Biomedical Laboratory, Faculty of Medicine, Udayana University, Bali. Analysis of the results of the examination and research data for normality-homogeneity was tested with the KS test and the prevalence ratio of AIF expression using the SPSS 20-version for windows independent t test to be presented in the form of tables, narratives, and pictures. **Result:** 40 samples of preterm pregnancy were divided into two groups, namely 20 PROM and 20 without PROM, where the KS test did not show any significant differences in the variables of maternal age, parity, PROM, and income ( $p > 0.05$ ), except that the mean number of leukocytes was higher in the without PROM group with a previous history of PROM than in the PROM group ( $p < 0.05$ ). The mean total score of AIF expression in the PROM groups and without PROM groups were  $56.60 \pm 11.20$  and  $24.10 \pm 5.70$ , respectively, with a value of  $t = 11.60$ ;  $df = 28.20$ ;  $p = 0.001$ . **Conclusion:** Thus, it has been proven that there are differences in the AIF expression of amniotic cells in PROM cases and without PROM cases in preterm delivery.*

**Keywords:** AIF, PROM, preterm delivery

## 1. Introduction

Until now, premature rupture of membranes (PROM) in preterm pregnancy is still a maternal health problem related to prevalence, perinatal morbidity and mortality and maternal morbidity.

The prevalence of PROM in the world is between 5-15% of all pregnancies.<sup>1</sup> Whereas in China, the prevalence of PROM is 19.53% of all pregnancies.<sup>2</sup> Dars et al. reported that the prevalence of preterm PROM is about 3% of pregnancies and this accounts for 33.0% of preterm births and 40-75% of neonatal deaths.<sup>3</sup> In Indonesia, the prevalence of PROM ranges from 4.5-7.6%. Meanwhile, Budijaya and Negara reported that the prevalence of PROM was 14.62% (212/1450) of deliveries comprising 84.43% term and 15.57% preterm.<sup>4</sup>

Rupture of the amniotic membrane with no sign of labor that causes the spontaneous discharge of amniotic fluid, which is known as premature rupture of membranes (PROM), is the most common cause of infection during pregnancy. This is thought to be related to changes in biochemical processes that occur in the extracellular matrix collagen amnion, chorion, and fetal membrane apoptosis processes. Until now, the exact cause of PROM is still not agreed and suspected to be multifactorial. Some factors reported include infection, hormonal, mechanical (multiple pregnancies,

polyhydramnios, and large children), low socioeconomic conditions, and apoptosis of the amniotic epithelial cells.

Recently, apoptosis has received widespread attention regarding the risk of rupture of the membranes; especially in preterm pregnancy, which shows an imbalance between proapoptotic protein and antiapoptotic protein.<sup>5</sup>

Apoptosis is thought to be associated with PROM because of finding apoptotic cells in the area adjacent to the tear of the amniotic membrane; called the paracervical weak zone so that this zone is significantly weaker than the other zones. Various studies have provided consistent results that the amniotic membrane of pregnant women with PROM shows higher apoptosis compared to the membranes of both terms and preterm labor with intact membranes.<sup>5,8</sup>

The apoptosis mechanism occurs through two main routes, namely caspase dependent and caspase independent. The first pathway, namely caspase dependent, can be an intrinsic pathway that is triggered by mitochondrial metabolic failure and an extrinsic pathway triggered by death receptors. Meanwhile, the second pathway, caspase independent, is triggered by mitochondrial proteins where the role of apoptosis inducing factor (AIF) is thought to be very important; even dominant.<sup>6</sup>

The apoptosis is thought to be initiated by proteins in the mitochondria of the epithelial cells of the amniotic membrane itself; especially apoptosis inducing factor (AIF). This protein is a flavoprotein with nicotinamide adenosine dinucleotide hydrogen (NADH) oxidase activity, which is found in mitochondria.<sup>7</sup> Current studies only report on the caspase dependent apoptotic pathway associated with PROM events and a little through caspase independent. As described, the AIF-mediated caspase independent mechanism plays an important role in the induction of apoptosis which weakens the structure of the amniotic membrane so it ruptures easily. However, the research sources related to AIF and PROM are still very minimal.

Thus, the role of apoptosis of the amniotic membrane in PROM pregnancy and preterm labor needs further evaluation. The indicator of apoptosis is the expression of AIF of the amniotic membrane which is the material for examination in preterm pregnancy which ends in labor. The purpose of this study was to determine the role of AIF expression of amniotic cells in the mechanism of PROM in preterm labor.

## 2. Methods

### 2.1 Research design and population

This study used an observational analytic cross sectional design. The sampling technique used consecutive sampling. Subjects who are willing to take part in the research will be checked for AIF expression by the Integrated Biomedical Laboratory at the Faculty of Medicine, University of Udayana. The population in this study were pregnant women with a gestational age of less than 37 weeks and experienced premature rupture of membranes, then the comparison group was women who delivered at full term without premature rupture of membranes.

The inclusion criteria in this study were gestational age less than 37 weeks, had been treated with premature labor and was accompanied by premature rupture of membranes, pregnancy with a live single fetus, no signs of systemic infection in the mother, and willingness to take part in the study after obtaining an informed consent. The exclusion criteria in this study were polyhydramnios, tingling, macrosomia in the fetus, history of coitus within 24 hours, smoking patients, having malignancy, patients with congenital disorders such as Marfan syndrome.

### 2.2 Immunohistochemical assessment of amniotic cells

The expression of AIF is the intensity of the brown area in the cytoplasm stained by immunohistochemical examination. The intensity of the brown color is then semi-quantitative by calculating the percentage of the cell cytoplasm that is recorded. The immunolabeling intensity was given a score of 0 (no stained cells), 1+ (stained <25% positive staining), 2+ (moderate intensity 25-50% stained), and 3+ (high intensity > 50% stained).

### 2.3 Data analysis

Data analysis in this study used the SPSS version 20.0 program. Numeric variables will be presented in terms of mean and standard deviation, while categorical variables will be described based on the number and percentage. The normality test is used to examine the normal distribution of the variables in this study. Independent t-test was used to compare AIF expression in the PROM groups and without PROM groups and to compare individual characteristics such as age, parity, body mass index, and leukocyte count. If the distribution of data is not normal, the Mann Whitney-U non-parametric test is used. Categorical variables will be tested using the Chi-square test. All values are considered significant if  $p < 0.05$ .

## 3. Research Result

### Distribution of Characteristics of Age, Parity, PROM, Leukocytes, Income, and History of PROM in the Two Groups

This cross-sectional observational study involved 40 preterm pregnancy samples comprising 20 as the preterm group with PROM and 20 as the preterm group without PROM in the Maternity Room, Emergency Room, Sanglah Hospital Denpasar. IHC AIF examination material with Kit (DakoEnVision® + Dual Link System-HRP (DAB +) is amniotic tissue taken from the amniotic membrane on the torn side about 3 x 3 cm after delivery, put in a sterile phosphate buffer saline (PBS) transport medium and examined at the Integrated Biomedical Laboratory, Faculty of Medicine, University of Udayana Denpasar.

In this study, we carried a comparative test out on the variables of age, parity, PROM, leukocytes, income, and history of PROM between subjects from the two groups. The analysis showed that there was no difference in characteristics between the two groups ( $p > 0.05$ ).

**Table 1:** Distribution of Characteristics of Age, Parity, PROM, Leukocytes, Income, and History of PROM in the Two Groups

Characteristics	Preterm Group		p
	With PROM (n = 20)	Without PROM (n = 20)	
Age (median, min-max)	27.5 (14-39)	22.5 (18-41)	0.211 **
Parity (median, min-max)	0 (0-3)	0 (0-3)	0.398 **
BMI (kg / m <sup>2</sup> )	22.27 (1.89)	21.59 (1.76)	0.247 *
Leukocytes (thousand)	15.09 (3.65)	18.65 (5.8)	0.027 *
Income (n)			
Low	4	5	0.208 ***
Moderate	11	14	
High	5	1	
Previous PROM history (n)			
Positive	19	9	0.001 ***
Negative	1	11	

Unpaired T-test \*

Mann-Whitney Test \*\*

Chi-square test \*\*\*

**Difference Expression of AIF in the Preterm Group with PROM and Preterm without PROM**

The results of the AIF expression examination from histochemical examination were expressed in the intensity and number of cells that expressed AIF in view with a microscope magnification of 400x. Based on the intensity of AIF expression in each cell, it was found that the PROM preterm group mostly expressed AIF (77.7%) with 41.6% high intensity AIF expression, while the preterm group without PROM groups only 48.9% expressed AIF (Table 2).

**Table 2:** Distribution of AIF Expression of Amniotic Epithelial Cells in the Two Groups

	Preterm with PROM		Preterm without PROM	
	Cell mean ± SD	Proportion of Total Cells * (%)	Cell mean ± SD	Proportion of Total Cells * (%)
Amniotic epithelial cells expressing AIF	23.1 ± 4.8	77.7	16.9 ± 3.4	48.9
Weak intensity (<25% stain positive)	1.8 ± 1.4	5.8	10.0 ± 2.3	29.4
Medium intensity (25-50% tint positive)	9.0 ± 2.8	30.2	6.5 ± 2.3	18.8
High intensity (> 50% positive color)	12.3 ± 2.5	41.6	0.4 ± 0.5	1.0
Amniotic epithelial cells do not express AIF	6.4 ± 2.4	22.3	17.8 ± 4.3	51.1
<b>Total Score of AIF Expressions **</b>	56.6 ± 11.2		24.1 ± 5.7	

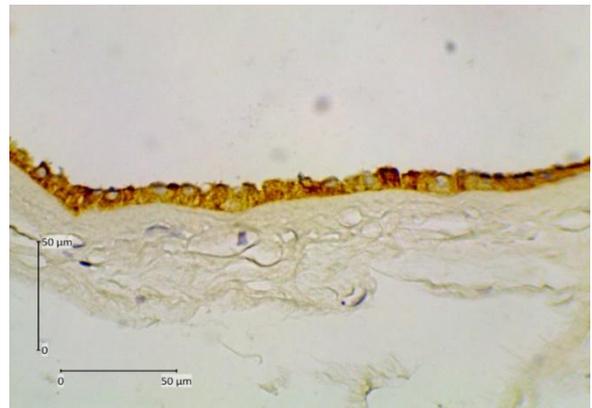
\* Total cells per field of view at 400X magnification  
 \*\* Total score is calculated by the accumulated score of each sample by adding up the intensity score of the AIF expression in each sample. The respective scores are weak intensity (1), medium intensity (2) and high intensity (3).

**Table 3:** Differences in AIF Expression of Amniotic Epithelial Cells in the Preterm and PROM Groups and the Preterm Groups without PROM Groups

	Preterm group with PROM		Preterm Group without PROM		t	df	p
	Average	SD	Average	SD			
AIF expression	56.6	11.2	24.1	5.7	11.60	28.2	0.001

**Microscopic Overview of AIF Expression in Amniotic Cells**

From the immunohistochemical examination, the AIF expression of the amniotic cells showed reddish cytoplasm staining with a brownish nucleus which showed positive AIF expression as shown in Figure 1 and bluish in negative AIF expression as shown in Figure 2. The intensity was brown then semiquantitative by calculating the percentage of the cell cytoplasm that was stained. Immunolabeling intensity was scored as 0, 1+, 2+, and 3+. Then these scores are grouped into two, namely the AIF expression (+) if the score is 1+, 2+ or 3+ and the AIF expression is (-) if the score is 0.



**Figure 1:** Expression of Positive AIF in Amniotic Cells (400 times Magnification)



**Figure 2:** Expression of Negative AIF in Amniotic Cells (400 times Magnification)

**4. Discussion**

Based on the results, there is a difference between the AIF expression of amniotic cells and the occurrence of premature rupture of membranes in preterm labor, where 77.7% of preterm labor with PROM group expressed AIF with 41.6% high-intensity AIF expression, while the preterm group without PROM only 48.9% who expressed AIF.

The results of the independent T-test mean score of AIF expression in the preterm labor group with PROM (56.6 ± 11.2) and the preterm delivery group without PROM (24.1 ± 5.7) resulted in a value of T = 11.60; df = 28,2; p = 0.000. Statistically, these results can accept the research hypothesis that there is a difference in the mean score of AIF expression on amniotic cells between PROM cases and without PROM in preterm labor.

Country et al. previously conducted research related to the relationship between AIF expression and the occurrence of PROM. However, the study included subjects with preterm and term labor (20-42 weeks' gestation), where the results of the study reported that AIF had a strong association as a risk factor for developing PROM.9

Country et al. also conducted research related to AIF expression on the risk of PROM and obtained similar results where AIF expression was proven to be a risk factor for the

occurrence of PROM with OR of 6.60 (CI 95% = 1.48-29.36;  $p = 0.009$ ).<sup>10</sup>

The apoptosis mechanism through the independent caspase pathway does not require caspase intermediaries and has its own mechanism for cell death. Apoptosis in this independent caspase pathway that plays a role is the mitochondrial pro-apoptosis protein molecule, namely Apoptosis Inducing Factor (AIF) and Endonuclease G.<sup>6</sup>

Apoptosis Inducing Factor is a flavoprotein with nicotinamide adenosine dinucleotide hydrogen (NADH) oxidase activity normally found in mitochondria.<sup>7</sup> AIF was discovered to be the first protein to regulate the apoptosis mechanism in a caspase-independent manner. AIF functions as NADH oxidase and uses FAD as a cofactor. In healthy cells, AIF is bound N-terminal to the inner mitochondrial membrane. On receiving the apoptotic signal, AIF is broken down from the inner mitochondrial membrane, released into the cytosol, transmitted into the nucleus, binds to DNA and causes condensation and expansion of chromatin. In addition, cytosolic AIF can also trigger the release of more AIF from the mitochondria, which causes further damage to the mitochondria and exacerbates AIF leakage.

Oxidative stress and DNA damage lead to activation of Poly ADP ribose polymerase-1 (PARP = 1), a nuclear enzyme that synthesizes poly ADP ribose (PAR) at the expense of ATP and NAD<sup>+</sup>. Active PARP can trigger apoptosis through a variety of pathways, including through AIF activation. Therefore, some researchers state that AIF leakage can be caspase dependent or caspase-independent depending on the context.

The mechanism of premature rupture of membranes caused by infection of the genital tract can be in the form of extracellular bacterial infection or intracellular obligate bacteria. Genital tract infection can cause apoptosis of amniotic cells, where extracellular bacterial infection can pass through the caspase-dependent pathway and intracellular obligate bacterial infection through the independent caspase pathway. In the caspase dependent pathway, it can be seen by the presence of the caspase-3 parameter and the independent caspase path with the AIF parameter.

Obligate intracellular bacterial infection can damage mitochondria, so it can accelerate the apoptotic mechanism of the amniotic membrane through an independent pathway through increasing Bax protein expression and continues to activate AIF and Endonuclease G. In this condition, Bcl-2, which has a function to inhibit the permeability of the mitochondrial membrane, is inhibited. So that the Bax protein (a member of the Bcl-2 family) increases, causing the mitochondrial membrane pores to open, then causing AIF and Endonuclease G to translocate from the mitochondrial inter-membrane space to the nucleus to induce DNA fragmentation. This situation causes an increase in p53 which will end with the apoptosis process of the amniotic membrane.<sup>12,13</sup>

In both preterm and term PROM cases, accelerated apoptotic processes were reported, especially in areas of rupture of the

amniotic membrane.<sup>14</sup> The exact role of AIF in the incidence of PROM, especially in preterm delivery is still unknown. Until now, most of the existing studies only discussed the role of apoptosis, especially those mediated by the caspase dependent pathway. Meanwhile, literature regarding the caspase independent pathway and the role of AIF in cases of preterm PROM in pregnant women is still very limited.

The important role of AIF as a risk factor for PROM, especially in preterm delivery, has been demonstrated in this study. Where the discovery of apoptotic proteins in the form of AIF expression of amniotic cells, shows that there is indeed a relationship between AIF expression, as a parameter of the presence of apoptosis through the caspase independent pathway on the amniotic membrane, with the incidence of PROM.

## 5. Conclusion

Based on the results of this study, it can be concluded that there are differences in the AIF expression of amniotic cells in preterm labor with PROM and without PROM ( $t = 11.60$ ,  $df = 28.20$ ,  $p = 0.001$ ). 77.7% of the preterm labor group with PROM expressed AIF, while the preterm group without PROM only 48.9% expressed AIF.

### Conflict of interest

The author states that there is no conflict of interest regarding the publication of this research.

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### Ethics in research

This research has received the approval of the Research Ethics Commission of the Faculty of Medicine, Udayana University / Sanglah Hospital Denpasar, dated July 9, 2019, No 2051 / UN14.2.2.VII.14 / LP / 2019 and Research Permit dated 18 October 2019, No LB.02.01 / XIV. 2.2.1 / 39619/2019.

## References

- [1] Getahun D, Stricland D, Ananth C, Fasseth M, Kirby S, Jacobsen S. Recurrent of Preterm Rupture Of Membranes In Relation To Interval Between Pregnancies. *American Journal of Obstetrics And Gynecology*. 2010; 220: 570.e1-6.
- [2] Liu J, Zhi-Chun F, Jing W. The Incidence Rate of Premature Rupture of Membranes and its Influence on Fetal – neonatal Health: A Report From Mainland China. *Journal of Tropical Pediatrics*. 2010; 56 (1): 36–42.
- [3] Dars S, Malik S, Samreen I, Kasi RA. Maternal morbidity and Perinatal Outcome in Preterm Premature Rupture of Membranes before 37 weeks gestation. *Pakistan Journal of Medical Sciences*. 2014; 30 (3): 626.
- [4] Budijaya M, State SNK. Profile of childbirth with premature rupture of membranes in Sanglah General Hospital, Denpasar for the period of January 1 -

- December 31 2015. Obstetrics and Gynecology Section / SMF, FK Unud / RSUP Sanglah Denpasar; 2016.
- [5] Cunningham FG. Preterm Birth. In: Williams 23rd Obstetrics. Ed. United States of American: McGraw-Hill. 804-831; 2010.
- [6] Elmore S. Apoptosis: A Review of Programmed Cell Death. *Toxicologic Pathology*. 2007; 35: 495–516.
- [7] Brown D, Yu BD, Joza N, Bénit P, Meneses J, Firpo M, Rustin P, Penninger JM, Marti GR. Loss of Aif Function Causes Cell Death In The Mouse Embryo, But The Temporal Progression of Patterning Is Normal. *PNAS*. 2006; 103 (26): 9918-9923.
- [8] SNK countries, Surya I, Sanjaya H, Anantasika A, Mahardika I. Lower Serum Catalase Level is Associated with Preterm Labor among Pregnant Women at Sanglah Hospital Denpasar, Bali-Indonesia. *Bali Medical Journal*. 2016; 5 (3): 404-407. DOI: 10.15562 / bmj.v5i3.292
- [9] SNK countries, Suwiyoga K, Arijana K. Role of Apoptosis Inducing Factor (AIF) as risk factors of premature rupture of membranes. *Biomedical and Pharmacology Journal*. 2018; 11 (2): 719–724. doi: 10.13005 / bpj / 1425.
- [10] SNK Countries, Suwiyoga K, TGA Payers. The Role of Caspase-3, Apoptosis-Inducing Factor, and B-cell Lymphoma-2 Expressions in Term Premature Rupture of Membrane ', *Revista Brasileira de Ginecologia e Obstetricia*. 2018; 40 (12): 733–739. doi: 10.1055 / s-0038-1675611.
- [11] Prabhu SB, Khalsa JK, Banerjee H, Das A, Srivastava S, Mattoo HR, Thygarajan K, Tanwar S, Das DS, Majumdar SS, George A, Bal V, Durdik JM, Rath S. Role of apoptosis-inducing factor (AIF) in the T cell lineage. *Indian J Med Res*. 2013; 138: 577-590.
- [12] Gao LY, Kwaik YES. The modulation of host cell apoptosis by intracellular bacterial pathogens. *Trends In Microbiology*. 2000; 8 (7): 306-313.
- [13] Saglam A, Ozgur C, Derwig E, Unlu BS, Gode F, Mungan T. The Role of Apoptosis in Preterm Premature Rupture of the Human Fetal Membranes. *Arch Gynecol Obstet*. 2000; 288 (3): 501-5.
- [14] Menon R, Fortunato SJ. The Role of Matrix Degrading Enzymes in Rupture of Membrane. *J Soc Gynecol Investig*. 2009; 11 (7): 427-37.
- [15] Prabantoro B, Askandar M. The Role of Endonuclease G as a Determinant Biomarker of Amnionic Cell Apoptosis in Pregnancy with Premature Rupture of Membrane. *JBP*. 2011; 13: 27-37.