

## Apoptotic Index of Amniotic Membrane Cells in Preterm Labor with Premature Rupture of Membrane (PPROM) was Higher than Preterm Labor without Premature Rupture of Membrane

Giri Chandra<sup>1\*</sup>, Ketut Suwiyoga<sup>1</sup>, Ketut Surya Negara<sup>1</sup>, I Gede Mega Putra<sup>1</sup>, I Nyoman Hariyasa Sanjaya<sup>1</sup>, I Gede Ngurah Harry Wijaya Surya<sup>1</sup>, I Gusti Kamasan Nyoman Arijana<sup>2</sup>, Ketut Tunas<sup>3</sup>

<sup>1</sup>Department of Obstetrics and Gynecology, Universitas Udayana/Sanglah General Hospital, Bali.

<sup>2</sup>Integrated Biomedical Laboratory, Faculty of Medicine, Universitas Udayana, Bali.

<sup>3</sup>Department of Public Health, Universitas Dhyana Pura, Bali.

\*Corresponding Author: **Giri Chandra**

### Abstract

**Introduction:** Premature rupture of the membrane in preterm pregnancy is a condition that can cause increased maternal and perinatal morbidity and mortality. The study aims to compare the apoptotic index of amniotic membrane cells in preterm labor with premature rupture of membrane (PPROM) and without premature rupture of membrane. **Method:** This is a cross sectional study conducted at the Department of Obstetrics and Gynecology, Sanglah Hospital Bali. The Amniotic membranes were taken from the placenta after delivery then apoptotic index was measured by TUNEL examination. **Result:** The study included each 20 women from both groups. There was no significant difference of maternal age, gestational age, and BMI between groups ( $p>0,05$ ). Chi-square analysis showed there was an association between the apoptotic index and preterm labor with PROM ( $p = 0.001$ ). Subsequent analysis resulted in prevalence ratio of 19.0 (95% CI = 2.81 - 128.6). **Conclusion:** This study concluded that the apoptotic index of amniotic membrane cells in PPRM was higher than preterm labor without premature rupture of membrane.

**Keywords:** *Apoptotic index, Preterm Premature Rupture of Membrane, PPRM.*

### Introduction

Premature rupture of membrane (PROM) defined as spontaneous rupture of the membrane before the onset of labor and are not followed by early signs of labor within one hour. Premature rupture of membrane still a common problem that contributes to poor pregnancy outcome.

If the premature rupture of the membrane occurs in preterm pregnancy (PPROM), it will require a significantly higher cost for immediate and long-term care of the preterm baby. Therefore, improving the understanding of risk factors, pathophysiology, and diagnosis of premature rupture of membranes in preterm pregnancy is one of the efforts to reduce both to maternal and perinatal morbidity and mortality.

According to the 2012 Indonesian Health Demographic Survey (IHDS), the Infant Mortality Rate (IMR) is 32/1000 live births, and a neonatal mortality rate is 19/1000 live births [1]. According to Getahun et al., the incidence of premature rupture of membranes ranges from 5% to 15% of all pregnancies [2]. Based on data from Osaikhuwoman, the incidence of premature rupture of membranes were around 4.5% to 7.6% of all pregnancies [3].

At Sanglah General Hospital Denpasar Bali, Indonesia, the incidence of premature rupture of the membrane was 12.92 % of 2113 deliveries, in which cases term premature rupture of the membrane was 83.23 % and preterm premature rupture of the membrane was 16.77 % [4].

The functions of the fetal membrane are to maintain and guarantee the fetal nutritional needs during pregnancy [5]. Maintenance of the integrity of the membrane during pregnancy is necessary for fetal development [6]. However, in the premature rupture of membrane, occurs cell apoptosis. In cell apoptosis, there is an increase in the breakdown of ADP-ribose polymerase I, decrease tissue inhibitors of metalloproteinase 3 (TIMP-3) and histological changes in the cell [7]. Cells that undergo apoptosis then formed apoptotic bodies and could be detected using TUNEL examination. Previous studies have shown that the membrane in labor with premature rupture of the membrane has a higher apoptotic index compared with the preterm labor without premature rupture of membrane [8].

Many similar studies regarding the apoptotic index led researchers to conduct a study on the association of the apoptotic index with the incidence of premature rupture of the membrane in preterm labor. Hopefully, this study could improve the growing bodies of evidence and stimulate a novel obstetric approach to reduce morbidity and mortality.

## Methods

This is a cross sectional study conducted at Department of Obstetrics and Gynecology, Sanglah Hospital Bali and Integrated Biomedical Laboratory of Medical Faculty, Universitas Udayana, Bali from July 2017 until February 2018. Study samples were pregnant women with 20 weeks until 36 weeks six days gestational age who admitted to the Sanglah Hospital Bali that met the inclusion and exclusion criteria and was willing to participate in the study after signed on informed consent.

Inclusion criteria were pregnant women with 20 weeks until 36 weeks six days gestational age, singleton live fetus, no maternal infection. Exclusion criteria were multiple pregnancies, polyhydramnion, hypertension in pregnancy or decline to participate in the study. A total of 40 pregnant women were included and consisted of 20 pregnant women

with PROM and 20 preterm pregnant women without PROM.

## Amniotic Membrane Processing

The amniotic layers of fetal membranes were taken from the placenta as soon as the preterm labor completed. Amniotic membranes were collected in equals size from the ruptured part, each 2 x 3 cm in size. Then, the samples were fixated into phosphate-buffered saline (PBS) solution. The tissue was processed into paraffin block then continued with deparaffinized and rehydrated using a series of graded alcohols (100%, 95%, 90%, 80%, 70 %) then rinsed with phosphate-buffered saline twice.

Tissue section on slide incubated for 15-30 minutes in Proteinase K solution at 21-37°C. The slide then incubated for 10 minutes in 3% H<sub>2</sub>O<sub>2</sub> and methanol at 15-25°C. Slide rinsed with phosphate-buffered saline, let it dried [9,10].

After the process of deparaffinization and rehydration was done, TUNEL's reagent was added to the slide and incubated for 60 minutes at 37°C. Then, the slide rinsed with phosphate-buffered saline three times. Streptavidin - HRP solution (50µl) added into the slide and incubated for 30 minutes at 37°C, and then slide rinsed with phosphate-buffered saline three times. Diaminobenzidine 10mg/ml and 30% H<sub>2</sub>O<sub>2</sub> (1µl) added into the slide (DAB) then slide rinsed with phosphate-buffered saline three times. After it dried, the samples were analyzed under 400x magnification of light microscope [11].

## Apoptotic Index Measurement

Apoptotic index of the amniotic cell defined as the number of apoptotic cell nuclei presence among 100 cells observed under 400x magnification of microscope (equation 1). Apoptotic index observed at the area which is contained the densest apoptotic nuclei on staining with TUNEL's method. High apoptotic index defined as >10% and low apoptotic index defined if ≤ 10% of apoptotic nuclei from 100 cells observed.

$$\text{Apoptotic Index} = \frac{\text{The number of apoptotic nuclei} \times 100 \%}{\text{The number of observed nuclei}} \dots\dots\dots (\text{Eq. 1})$$

## Statistical Analysis

The sample characteristics including age, gestational age, parity and body mass index were analyzed descriptively and, Shapiro-Wilk test to determine the distribution of data and mean comparison analysis based on the distribution of the data. The apoptotic index was analyzed by measure the Prevalence Ratio (PR) and Chi-Square test to determine the possible association of apoptotic index in preterm labor with PROM compared with preterm labor without PROM.

## Results

**Table 1 The Characteristics of Age, Gestational Age and BMI of PPROM and Preterm without PROM**

Risk Factors	Premature Rupture of Membrane Group (n=20)		Without Premature Rupture of Membrane Group (n=20)		p
	Mean	SD	Mean	SD	
Age (year)	27.65	7.34	25.10	7.10	1.000
Gestational age (weeks)	32.60	3.40	32.18	3.38	1.004
BMI (kg/m <sup>2</sup> )	22.27	1.88	21.61	1.73	0.912

In Table 1 above, the maternal age, gestational age and body mass index obtained  $p$ -value  $> 0.05$ . This result showed that there were no significant differences in general characteristic between both groups.

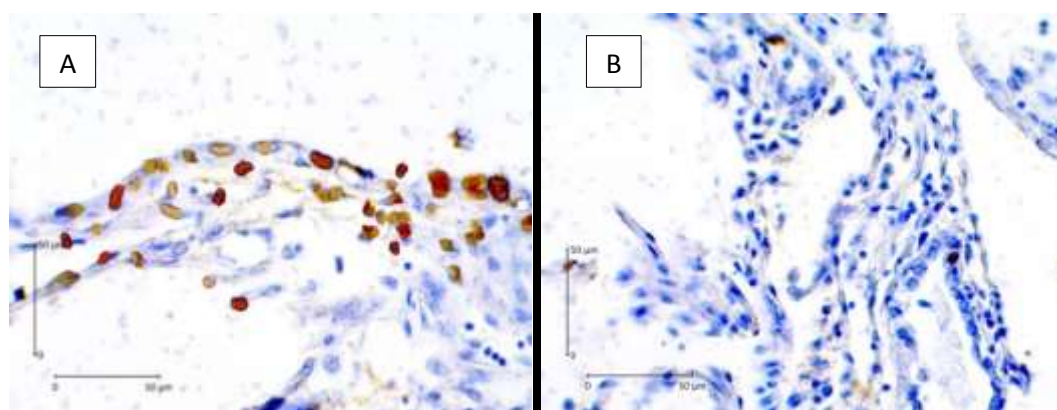
The association between the apoptotic index and the presence of premature rupture of the membrane in preterm labor were performed with the Chi-Square test and the effect was measured as Prevalence Ratio. The test results are presented in Table 2.

**Table 2 The Apoptotic Index of Amniotic Cells in PPROM and Preterm without PROM**

Group	Apoptotic Index of Amniotic cells		Prevalence Ratio	p
	High	Low		
Preterm PROM	19	1	19.0	95% CI = 2.81 - 128.6, $p = 0.001$
Preterm without PROM	1	19	128.6	

In Table 2 above showed the distribution of the apoptotic index, the prevalence ratio and the  $p$ -value obtained from Chi-square test. The Chi-square test and Prevalence Ratio

result were significant, PR = 19.0 (95% CI = 2.81 - 128.6,  $p = 0.001$ ). Prevalence Ratio showed that there was a strong association between PPROM and the high apoptotic index of amniotic cells.



**Fig. 1: Apoptotic Index Sampling of Preterm PROM (left) and Preterm without PROM (right)**

The Figure above (Figure 1) showed the result of the apoptotic index of amniotic cells examined by TUNEL method under 400x magnification of the microscope. The image on the left (Sample number 8 of PPRM) showed high apoptotic index (38%). In contrast, the right image (sample number 14 of Preterm Without PROM) had a low apoptotic index (2%).

## Discussion

Preterm PROM is a major complication of pregnancies. Currently, there is no practical way for fully preventing spontaneous rupture of fetal membranes, therefore, inability to control its incidence. It was partly due to yet fully known etiology and pathophysiology. This study was fueled by our institutions daily challenge. Sanglah General Hospital is a tertiary referral hospital and receives a high number of preterm labors with or without PROM. The previous study at Sanglah General Hospital Bali in 2015 and 2017 revealed that preterm PROM was high, which accounted for almost 15% of labor [12, 13].

By comparison, the study by Vishwakarma et al. obtained data on the premature rupture of the membrane was account for 3,75% of all labor and 17,6% of them were PPRM [14]. The almost similar result showed by a study by Okeke et al. obtained 3.3% of preterm premature rupture of membrane [15]. Due to the high number of cases and in effort to shed light on its etiology, this study was aimed to present evidence that premature rupture of the membrane in preterm pregnancy could be influenced by the intrinsic factors, not any other factor that had been excluded in analysis or from the general characteristic.

The characteristic of samples involved in this study had no significant differences in terms of maternal age, gestational age, and body mass index between the PPRM group and preterm without PROM. In this study, the average mother's age of group with PPRM was 27.65 years old, whereas in preterm mothers without PROM was 25.10 years old and was not significantly different ( $p$ -value = 1.00). The previous study by Surya Negara also showed a similar result. The average age of the mother in case PPRM was 27.67 years old while in the control group was 27.95 years old ( $p$ -value = 0.898) [13]. Our result also aligned with a study by Okeke et al. that showed the incidence of PPRM

occurs mostly within the 26-30 years old, as they found 43% of PPRM in this age range [15]. A similar study by Singh et al. showed that the incidence of preterm premature rupture of membranes was common in the 20-30 years old age group [16]. The gestational age between PPRM and preterm labor without PROM was also not significantly different ( $p$ -value = 1.004).

The result of the body mass index comparison between PPRM and preterm labor without PROM was also insignificant ( $p$ -value = 0.912). However, previous study by Thombre showed that PPRM was associated with increased body weight during the second and third trimester of pregnancy less than 0.37 kg in a week and a low body mass index before pregnancy, which is BMI <19.5 kg/m<sup>2</sup> [17]. Other studies by Kovavisarach stated that maternal body mass index associated with premature rupture of membrane if BMI <20.00 kg/m<sup>2</sup> [18].

Our study showed that there was a striking difference between the apoptotic index of the membrane taken after the labor between both groups. In the group of preterm labor with PROM, from 20 samples, 19 samples had a high apoptotic index (>10%) and only one sample had a low apoptotic index (≤10%). Overall, the preterm labor with PROM the apoptotic index ranged 9-38%. In vice versa, the group of preterm labor without PROM, there was only one sample with a high apoptosis index and the rest (19) had a low apoptotic index. The preterm labor without PROM had the apoptotic index ranged between 1-15%. The Chi-Square test confirms the finding as it resulted in  $p$ -value = 0.001 ( $p$ <0.05), therefore the difference was significant.

In the preterm rupture of the membrane, apoptotic cell cause weakness in the membranes. Apoptosis is a programmed process of cells death, starting with chromatin condensation (cell shrinking) continued with fragmented membrane and then formed organelle-containing cell membranes called apoptosomes or apoptotic bodies. In PPRM, occurs DNA damage which induced p53 protein activation and then it induced Bax and inhibits Bcl-2 in mitochondria to activate Cytochrome c. A proapoptotic molecule caused a caspase initiation and an executory caspase will activate endonuclease G.

This leads to DNA fragmentation and apoptotic bodies were formed which used as an indication of cell apoptosis. In this study, the apoptotic bodies were detected by TUNEL examination with the result was the apoptotic index [19-20]

## Conclusion

Preterm premature rupture of the membrane associated with higher apoptotic index of the amniotic cell compared to preterm labor

## References

- 1 Kementrian Kesehatan Republik Indonesia. Profil Kesehatan Indonesia 2012. Jakarta; 2013.
- 2 Getahun D, Strickland D, Ananth CV, Fassett MJ, Sacks DA, Kirby RS (2010) Recurrence of preterm premature rupture of membranes in relation to interval between pregnancies. *Am J Obstet Gynecol.* 202(6):570.e1-570.e6.
- 3 Osaikhuwuomwan J (2010) Preterm Rupture of Membrane: The Vitamin C Factor. *Benin J Postgrad Med.*, 12(1):60-8.
- 4 Suwiyoga K, Budayasa A (2006) Risk Factors of Premature Rupture of The Membrane Related to Incidence of Neonatal Sepsis in Term Pregnancy. *Cermin Dunia Kedokteran.* 151:14-7.
- 5 Soewarto S (2012) Premature rupture of the membrane. In: *Obstetric science 5th ed.* Jakarta: PT Bina Pustaka Sarwono Prawirohardjo; 677-82.
- 6 Menon R, Fortunato SJ (2007) Infection and the role of inflammation in preterm premature rupture of the membranes. *Best Pract Res Clin Obstet Gynaecol.* 21(3):467-78.
- 7 Harirah HM, Borahay MA, Zaman W, Ahmed MS, Hankins GD (2012) Increased Apoptosis in Chorionic Trophoblasts of Human Fetal Membranes with Labor at Term. *Int J Clin Med.* 3(2):136-42.
- 8 Rangaswamy N, Kumar D, Moore RM, Mercer BM, Mansour JM, Redline R, et al. Weakening and Rupture of Human Fetal Membranes-Biochemistry and Biomechanics. In: Morrison J, editor. *Preterm Birth - Mother and Child.* InTech; 2012.
- 9 Dabbs D. *Diagnostic Immunohistochemistry: Theranostic and Genomic Applications.* 3<sup>rd</sup> Editio. Philadelphia: Elsevier Saunder; 2010. 807p.
- 10 Runic R (1998) Apoptosis and Fas Expression in Human Fetal Membranes. *J. Clin Endocrinol Metab.* 83(2):660-6.
- 11 Newbold A, Martin BP, Cullinane C, Bots M (2014) Detection of Apoptotic Cells Using Immunohistochemistry. *Cold Spring Harb Protoc.*, (11):pdb.prot082537.
- 12 Budijaya M, Negara K, Suwiyoga K. *Profil Persalinan dengan Ketuban Pecah Dini di RSUP Sanglah Denpasar Periode 1 Januari – 31 Desember 2015.* Universitas Udayana; 2016.
- 13 Negara KS, Suwiyoga K, Arijana K, Tunas K (2018) Role of Apoptosis Inducing Factor (AIF) as Risk Factors of Premature Rupture of Membranes. *Biomed Pharmacol J.*, 11(2):719-24.
- 14 Vishwakarma K, Patel SK, Yadav K, Pandey A (2015) Impact of premature rupture of membranes on maternal & neonatal health in Central India. *J Evid Based Med Healthc.*, 2(49):8505-8.
- 15 TC O, JO E, OS O, CO A, EC E, PU A (2014) The Incidence and Management Outcome of Preterm Premature Rupture of Membranes (PPROM) in a Tertiary Hospital in Nigeria. *Am. J. Clin. Med. Res.*, 2(1):14-7.
- 16 Singh TD, Usham R, Kamei H (2015) Preterm Labor Rupture of Membrane: 1 Year Study. *J Evol Med Dent Sci.*, 4(49):8495-8.
- 17 Thombre MK (2014) *A Review of The Etiology Epidemiology Prediction And Interventions of Preterm Premature Rupture of Membranes.* Michigan State University.
- 18 Kovavisarach E, Sermsak P (2000) Risk factors related to premature rupture of membranes in term pregnant women: a case-control study. *Aust N Z J Obstet Gynaecol.*, 40(1):30-2.

without premature rupture of the membrane.

## Acknowledgements

The authors thank all staff at the Department of Obstetrics and Gynecology Sanglah Hospital, Histology staff at the Integrated Biomedical Laboratory, Medical Faculty of Universitas Udayana and the staff of Department of Public Health, Universitas Dhyana Pura, Bali Indonesia, for their support in this study.

- 19 Menon R, Boldogh I, Hawkins HK, Woodson M, Polettini J, Syed TA (2014) Histological Evidence of Oxidative Stress and Premature Senescence in Preterm Premature Rupture of the Human Fetal Membranes Recapitulated in Vitro. *Am. J. Pathol.*, 184(6):1740-51.
- 20 Sojasi S, Gebreili M, Assadollahi M, Hosseini M, Jafarabadi M (2018) Determination and comparison of stomach residual volume at two alternate gavage ways with gravity and injection force in premature infants. *Bali Medical Journal*, 7(1):170-176.