Protein 53 (P53) Expressions and Apoptotic Index of Amniotic Membrane Cells in the Premature Rupture of Membranes

Ketut Surya Negara[1*], Ni Luh Lany Christina Prajawati[1], Gede Putu Surya[1], Suhendro Suhendro[2], Komang Arijana[3], Ketut Tunas[4]

[1]*Department of Obstetrics and Gynecology, Udayana University, Sanglah Hospital, Bali, Indonesia;* [2]*Department of Pathology, Sanglah Hospital, Bali, Indonesia;* [3]*Integrated Biomedical Laboratory, Medical Faculty of Udayana University, Bali, Indonesia;* [4]*Department of Public Health, Dhyana Pura University, Bali, Indonesia*

Abstract

BACKGROUND: The premature rupture of membranes (PROM) represents an obstetric issue causing significant maternal and neonatal morbidity and mortality. Although protein 53 (p53), one of the proapoptotic proteins suspected of causing PROM at the molecular level is closely correlated with the occurrence of PROM, the exact mechanism remains still unclear.

AIM: This study aims to investigate the hypothesis that p53 expression and the apoptotic index play a role in the PROM mechanism.

METHODS: Placentas from 20 pregnancies (37–42 weeks gestation) and 20 pregnancies complicated by PROM were collected at delivery. The independent variable is represented by pregnant mothers with a single live fetus experiencing PROM (followed by labour and birth) while without PROM mothers represent the control. The research material was taken from the amnion tissue in the placenta. Also, p53 and apoptotic index (TUNEL) immunohistochemical examination were conducted at the Integrated Biomedical Laboratory, Medical Faculty of Udayana University, Bali. The correlation between the apoptotic index and p53 expression of the PROM group was tested using a McNemar Test.

RESULTS: No statistically significant differences were found between the two groups (p > 0.05). There was a significant difference in p53 expression in PROM cases compared to those without PROM (11.15 + 5.59% vs. 0.95 + 2.52%) with χ^2 = 19.538 and p = 0.001. The apoptotic index in PROM cases was higher than in those without PROM (19.10 + 5.63% vs. 1.15 + 2.46%) with χ^2 = 32.40 and p = 0.001. There was a strong correlation between p53 expression and PROM with PR = 3.449 (95% CI = 1.801-6.605; p = 0.001). There was a strong correlation between the apoptotic index and PROM with PR = 19 (95% CI = 2.81-128.69; p = 0.001).

CONCLUSION: p53 expression and the apoptotic index of amniotic membrane cells in cases of PROM was higher than in those without PROM, there was a strong correlation between p53 expression and apoptotic index with the occurrence of PROM.

Introduction

The premature rupture of membranes (PROM) is one factor causing increased maternal & neonatal morbidity and mortality rates due to the complications it causes [1] [2]. PROM involves the rupture of the fetal membrane before signs of labour occur [1]. This may occur at the end of the gestational period 37-42 weeks (term) and also far in advance of the normal gestational period, at less than 37 weeks (preterm) [1].

The rupture of the fetal membrane during labour is caused by weakening of the membrane due to uterine contraction and continuous stretching. In general, this membrane ruptures during uterine contraction; however, 10% of ruptures occur before uterine contractions in term pregnancies, and 40% occur during preterm pregnancy. This finding supports the case that contractions causing stretching of the uterus are not the only factor causing PROM [3].

Other factors suspected of causing PROM to include infection, hormonal changes, mechanical forces, and apoptosis [4]. The latest evidence is that PROM occurs before uterine contractions begin which is connected with amniotic cell apoptosis through caspase-dependent pathways mediated by p53 [5].

The proapoptotic effects of increasing p53 expression through intrinsic pathways induce amniotic cell's apoptosis. The increasing p53 expression via the molecular approach becomes a factor which plays a role in PROM pathogenesis, and this represents a very promising area for future research [6] [7]. Indeed, p53 is one of the key proapoptotic proteins implicated at the molecular level as it has a close correlation with the incidence of PROM, although the exact mechanism remains unclear [8].

The present study aims to show p53 expression, and the apoptotic index plays a role in the PROM mechanism and correlates p53 expression and the apoptotic index with the occurrence of PROM.

Material and Methods

Amniotic membranes were collected from participants suffering from PROM (n = 20) and those without PROM (i.e. normal labour) (n = 20) at term gestational age (37-42 weeks). Samples were only taken from patients who had been both well informed and signed a consent form. Amniotic membranes were taken from the edge of a rupture (2 cm in size) after labour using aseptic methods and samples were then placed into phosphate-buffered saline (PBS) solution as a transport-fixation solution.

Tissue Processing for Preparation of p53 Immunohistochemistry (IHC) and TUNEL

Amniotic membrane tissue was histopathologically processed into a paraffin block which was then cut at 3 to 5 μm, mounted on positively charged slides, and dried in an oven at 60°C for 30 minutes to ensure adherence to the slide. The sections were deparaffinized in four changes of xylene for 5 minutes each and then rehydrated through a series of graded alcohol with a final rinse in distilled water [9].

Endogenous peroxides quenched by soaking the section in two changes of 0.3% H_2O_2 in methanol. The immunostaining used the p53 kit from Labvision[R]. The tissue section was treated with antigen retrieval and then incubated inside a steamer for 20 minutes. Initially, samples were heated to 99°C, then taken out of the steamer, and allowed to cool down for 20 minutes (still in the target retrieval solution). The slide was washed 3 times with distilled water and then put in a Tris-buffered saline solution for 5 minutes. The slide reacted with the p53 primary antibodies for 1 hour. The positive control was derived from human colonic tissue and PBS solution as a negative control. Streptavidin-biotin-based detection was used, then an antibody was added and incubated for 15 minutes, then diaminobenzidine (DAB) chromogen was added. Counterstaining with Harris-haematoxylin was performed for 1 minute. The slide was washed with 0.25% ammonia until it became blue and then washed with distilled water. The slide was dehydrated with graded alcohol and then cleared with xylol 4 times. Finally, it was mounted and covered with a coverslip [9] [10].

Protein 53 expressions were performed by counting the positive brown-stained nuclei on 200 epithelial and stromal nuclei of amniotic membrane cells at a hotspot area that expressed the highest number of apoptotic nuclei, and then the percentage was calculated [11]. Protein 53 expression was scored by the percentage and intensity of the staining: a score of 0 = negative; a score of 1+ = weak; a score 2+ = moderate; a score 3+ = strong; as presented in Table 1 [12] [13] [14].

Table 1: Interpretation of P53 Immunohistochemical Staining [14] [15]

Staining Pattern	Score
No nuclei staining or less than 10% nuclei staining	0
Staining more than 10% of nuclei, weak intensity, incomplete nuclei staining	1+
Staining more than 10% nuclei, weak to moderate intensity, complete nuclei staining	2+
Staining more than 10% nuclei, strong intensity, complete nuclei staining	3+

The normality of the data was tested using a Shapiro-Wilk Test, and homogeneity was tested by Levene's test. Protein 53 expression data were descriptively analysed using a Mann Whitney U-Test (due to abnormal distribution) to analyse the differences between the two groups. A Chi-Square Test was performed to analyse the correlation between p53 expression and apoptotic index with PROM. The correlation between the apoptotic index and p53 expression of the PROM group was tested using a McNemar Test. A significance level of $p < 0.05$ was considered significant.

After being deparaffinized and rehydrated, slides were then rinsed twice with PBS for two minutes each. The slides were incubated for 15-30 minutes at 21-37°C in a working solution Proteinase K (containing Proteinase K 2 μL in 98 μL PBS). The slides were then incubated in a blocking solution for ten minutes at 15-25°C (the blocking solution contained H_2O_2 3% in methanol). Slides were rinsed with PBS twice for two minutes each following the labelling protocol. Slides were rinsed with PBS twice for 2 minutes each, and then the area surrounding the samples was allowed to dry. The reagent of TUNEL was added to the samples, which were then covered and incubated for 60 minutes at 37°C in a dark humidity chamber. Slides were rinsed with PBS three times for two minutes each and then left to dry. Streptavidin-HRP solution (50 μL) was added to the

samples. Slides were incubated in a dark humidity chamber at 37°C for 30 minutes. Slides were rinsed with PBS three times for two minutes each. Then, 50-100 µL diaminobenzidine (DAB) substrate was added, and the slides were incubated for 10 minutes at 15-25°C (the DAB substrate contained 5 µL 10 mg/mL DAB buffer and 1 µL 30% H_2O_2 in µL PBS; freshly prepared). Slides were rinsed with PBS three times, covered with a coverslip, and analysed under a light microscope [9].

The apoptotic index of amniotic cells consists of the number of apoptotic amniotic cell nuclei in 100 cells observed under a microscope at 400 x magnification. The apoptotic index was observed by a pathologist at the hotspot area which contained the densest apoptotic nuclei on routine staining of haematoxylin-eosin (HE). The apoptotic index is determined as _strong_ if > 10%, and weak if $\leq$ 10% [17].

$$\text{Apoptotic Index} = \frac{\text{The number of apoptotic nuclei}}{\text{The number of observed nuclei}} * 100\%$$

Results

The average age of mothers in the PROM group was 27.15 years: primigravida had the first rank with 10 cases (50%), the average gestation age (GA) of the PROM group was 38.7 weeks, and the average of body-mass index (BMI) was 24.60 kg/m^2. The average age of the without PROM mothers' group was 27.80 years, multigravida (G2) had the first rank with 10 cases (50%), primigravida accounted for 7 cases (35%), the average GA of the without PROM groups was 39.3 weeks, and their average BMI was 24.60 kg/m^2. The statistical test results illustrate there were no significant differences in mother's ages, gestation ages, _gravida_, and BMI between the PROM and the without PROM groups with a significance of 0.777, 0.153, 0.515, and 1.000, respectively, which all significances higher than 0.05 (p > 0.05). The statistics on mother's ages, gestation ages, and BMI are presented in Table 2.

Table 2: The Subject Characteristics of PROM and the Without PROM Group

Characteristic	PROM Group (n = 20)		Without PROM Group (n = 20)		p
	Mean	SD	Mean	SD	
Mother's Age (year)	27.15	± 6.85	27.80	± 7.52	0.777
Gestational Age (weeks)	38.70	± 1.5	39.30	± 0.98	0.153
BMI (kg/m^2)	24.60	± 4.48	24.60	± 4.48	1.000

Twenty samples from the PROM group expressed p53 on epithelial and stromal cells of the amniotic membrane with a strong intensity in the 4-30% in range. In the 20 samples from the without PROM group, most of them exhibited a negative expression of p53; several samples expressed p53 with a weak intensity in the 1-11% in range. The data

on p53 expression in the PROM and without PROM groups are presented in Figure 1. The p53 expression in the PROM and without PROM groups data did not follow a normal distribution based on a normality test.

Table 3: Comparison of Amniotic Cells P53 Expression of PROM and Without PROM Group

Group	p53 Expression				χ^2	p
	Negative	Weak	Moderate	Strong		
PROM	7	0	2	11	19,538	0,001
PROM-free	19	1	0	0		

In the PROM group, 11 samples (55%) expressed strong p53; 2 samples (10%) expressed moderate p53, and 7 samples (35%) were absent of p53. In the without PROM groups, 1 sample (5%) expressed weak p53, and 19 samples (95%) were absent of p53. There were significant differences in p53 expression within the PROM group which was higher than in the without PROM groups (11.15 ± 5.59% vs 0.95 ± 2.52%) with p=0.001 using a Mann Whitney U-Test. The value of x^2 was 19.538 using a Chi-Square Test as presented in Table 3.

Table 4: Correlation between p53 Expression and PROM

Variable		PROM		PR	CI 95%	P
		Yes	No			
p53 Expression	Positive	13	1	3.449	1.801 – 6.605	0.001
	Negative	7	19			

There was a strong correlation between p53 expression and PROM with PR = 3.449 (CI95% = 1.801-6.605; p=0.001). This finding reveals that p53 was a proapoptotic protein that plays the role of a regulator of apoptosis through intrinsic pathways and had a strong correlation as a risk factor of PROM (PR > 1).

The PROM group had a strong apoptotic index based on 19 samples (95%) and a weak apoptotic index in 1 sample (5%). The without PROM groups had a strong apoptotic index based on 1 sample (5%) and a weak apoptotic index based on 19 samples (95%). The PROM group had an apoptotic index of 8-30%. Of the twenty samples from the without PROM groups, most of them had a weak amniotic-cell apoptotic index based on IHC TUNEL. One sample of the without PROM groups had a strong amniotic-cell apoptotic index, with a value of 11%. The amniotic cell's apoptotic index in the without PROM groups had a range of 0-11%. The data on the apoptotic index microscopy of the PROM and without PROM groups are presented in Figure 2.

There were significant differences between the apoptotic index of the PROM group which was higher than the without PROM groups (19.10 ± 5.63% vs 1.15 ± 2.46%) with x^2 = 32.40 and p = 0.001 using a Chi-Square and a Mann Whitney U-Test as presented in Table 6. There was a strong correlation between the apoptotic index and the occurrence of PROM with a value of PR = 19 (CI 95% = 2.81-128.69; p = 0.001) as presented in Table 7.

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Table 5: The Distribution Value of Amniotic Cells Apoptotic Index of the PROM Group and the without PROM groups

Variable	PROM Group (n = 20)		Without PROM Group (n = 20)		p
	Mean	SD	Mean	SD	
Apoptotic Index	19.10	5.63	1.15	2.46	0.001

The results of the Chi-Square Test revealed that the value of $\chi^2 = 32.40$ and p = 0.001. This finding shows there were significant differences in the amniotic-cell apoptotic index between the PROM group and the without PROM groups (p < 0.05). The apoptotic index of the PROM group was higher than the without PROM groups.

Table 6: The Differences between the Amniotic-Cell Apoptotic Index of the PROM and without PROM groups

Group	Amniotic Cells Apoptotic Index		χ^2	p
	Weak	Strong		
PROM	1	19	32.40	0.001
PROM-free	19	1		

The stronger p53 expression in the PROM group was also associated with a stronger apoptotic index compared to the PROM-free group.
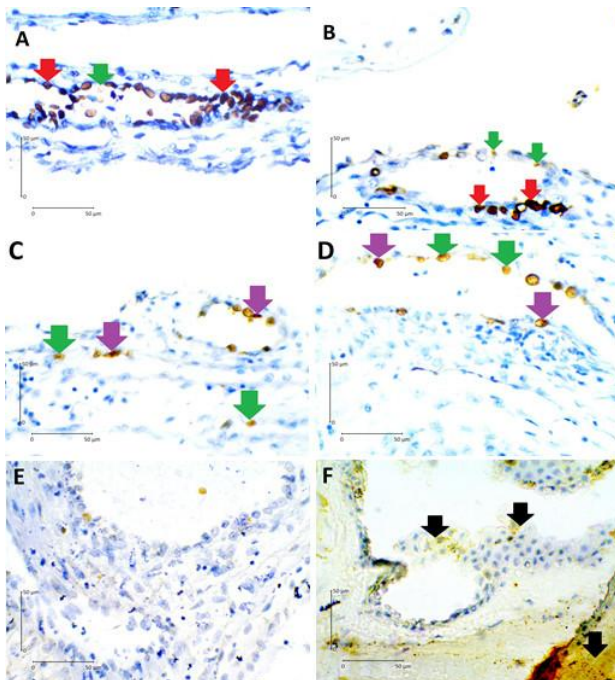

Figure 1: Protein 53 Expression in PROM and Without PROM Groups. A and B: Sample number 6 of the PROM group, p53 score 3+, strong expression (30%). Total 210 epithelial and stromal amniotic cells on A and B, 66 nuclei complete stained with strong intensity (red arrow), 4 nuclei incomplete stained with weak intensity (green arrow), and the others, 140 cells, negative. C and D: Sample number 15 of the without PROM groups, p53 score 3 1+, weak expression (11%). Total 200 epithelial and stromal amniotic cells on C and D, 8 nuclei incomplete stained with moderate intensity (purple arrow), 14 nuclei incomplete stained with weak intensity (green arrow), and the others, 178 nuclei, negative. E and F: Sample number 7 of the without PROM groups, p53 score 0, negative expression (0%). All of the 200 epithelial and stromal amniotic cells on E dan F were negative for p53 IHC. Figure F: artefact pool of residual IHC reagent brown on the part of the amniotic cell cytoplasm and the extracellular (black arrow), although there were no stained nuclei. 400 x magnification. Score 0: negative expression, score 1+: weak expression, score 2+: moderate expression, score 3+: strong expression

This result shows the stronger amniotic-cell p53 expression in the PROM group was caused by the higher apoptotic rate, which is mediated by p53 because the p53 expression has been confirmed by the amniotic-cell apoptotic index calculated using TUNEL-based methods.

Table 7: Correlation between Amniotic-Cell Apoptotic Index and PROM

Variable		PROM		PR	IC95%	p
		Yes	No			
Apoptotic Index	Strong	19	1	19.0	2.81 – 128.69	0.001
	Weak	1	19			

The result of a McNemar Test on the amniotic-cell apoptotic index and p53 expression in the PROM group shows a significance level 0.031 (p < 0,05), therefore, it can be concluded there was a correlation between a strong amniotic-cell apoptotic index and strong p53 expression in the PROM group at term pregnancy.
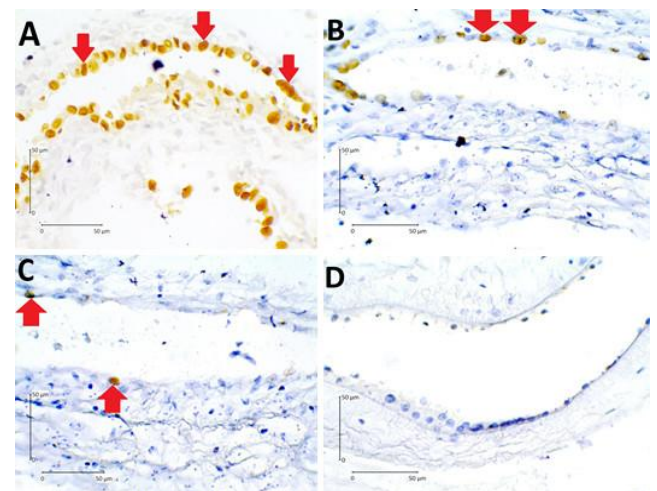

Figure 2: Apoptotic Index of PROM and without PROM Group. A) Sample number 6 of the PROM group. Strong apoptotic index, 30% (> 10%). From a total of 200 epithelial and stromal cells of amniotic membrane on one high power field (HPF), there were 60 nuclei with brown staining in TUNEL IHC (red arrow). B) Sample number 37 of the without PROM groups. Strong apoptotic index, 11% (> 10%). From a total of 100 epithelial and stromal cells of amniotic membrane on one HPF, there were 11 nuclei with brown staining in TUNEL IHC (red arrow). C) Sample number 31 of the without PROM groups. Weak apoptotic index, 2% (≤ 10%). From a total of 100 epithelial and stromal cells of amniotic membrane on 1 HPF, there were two nuclei with brown staining in TUNEL IHC (red arrow). D) Sample number 15 of the without PROM groups. Negative apoptotic index (0%). Total 50 epithelial and stromal cells of amniotic membrane on one HPF, there was no nuclei staining in TUNEL IHC. 400 x magnification. Strong apoptotic index: > 10% and weak apoptotic index: ≤ 10%

Discussion

The present study was conducted from January 2016-April 2018. Samples were taken by

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consecutive sampling from pregnant patients at Sanglah Hospital (and its educational facilities) in Denpasar, Bali, in 2016. Inclusion criteria were: 37-42 weeks GA, single live fetus, no sign of maternal infection, and the mother's agreement to participate in this study. Exclusion criteria were: a history of preterm PROM in a previous pregnancy, polyhydramnios, Gemelli, macrosomia, and sexual intercourse in the previous 24 hours. A previous study on the epidemiology of PROM at Sanglah Hospital in 2015 showed the prevalence of labour with PROM was 179 cases (83.43%), while preterm PROM was 33 cases (15.57%) [19]. A comparative study from India reported that the prevalence of PROM at term pregnancy was 82.1% while the prevalence of preterm PROM was 17.6 % [20]. The results of the present study at Sanglah Hospital (and those of the Indian study as a comparison) were no different to the prevalence in the variation of PROM cases globally; with PROM affecting a total of 5-10% cases of all labours [21]. The premature rupture of membrane represents an as-yet unsolved problem in obstetrics: PROM accounts for more than a 24-hour increase in maternal mortality (68%), higher neonatal mortality (37.5%), and neonatal death (2%) [22].

The characteristics of the current study's samples show no differences in mother's age, gestational age, BMI, and gravida between the PROM and without PROM groups. These findings are consistent with a previous study on PROM carried out at Sanglah Hospital which revealed significance regarding the comparison of mother's age, GA, and BMI in the PROM and PROM-free groups that showed no significant statistical differences with all values $p > 0.05$ [23].

The cases of PROM were higher in the primigravida subjects with 10 cases of PROM from a total of 20 cases, which accounts for 50% of PROM cases in this study. Primigravida in the without PROM groups were 7 cases from a total of 20, which accounts for 35% of the without PROM cases. Primigravida in the PROM group was more frequent compared with primigravida in the without PROM groups (50% vs 35%), although a statistical analysis using a Mann Whitney U Test shows a significance value of 0.453 ($p > 0.05$), which represents no significant difference. The 2015 study at Sanglah Hospital found that PROM is more prevalent in primigravida; accounting for 69 cases (32.55%) in 179 births [19]. The finding of this study is that there is no difference between primigravida in PROM and without PROM labour which is consistent with Patil _et al.,_ (2014), where the total cases of primigravida with PROM was 53 cases from 100 labours, compared with without PROM primigravida was 52 cases from 100 labours which are not a statistically significant difference [24]. The uteri of primigravida have a relatively low adaptation for gestation processes and so have a bigger risk of PROM.

Protein 53 expressions in the PROM group

was higher than in the without PROM groups. The mechanism functions mainly through the intrinsic pathway, with the mitochondria as the centre of the process. The role of p53 begins after its activation by several agents; then p53 activates Bax and inhibits Bcl-2. The main mechanism of Bax on mitochondrial is decreased permeability of the mitochondrial membrane caused by cytochrome c leaking and affecting the Ca^{2+} level by the forming of a channel when Bax collaborates with several types of Bax. This channel becomes the entry point of Ca^{2+} ions: when the ions enter, the cytochrome c moves from the mitochondria to the cytoplasm. The cytochrome c on the cytoplasm will be bonded to the _apoptosis-activating factor_ (apaf-1), a protein which is abutted or surrounded by Bcl-2 on the mitochondria's outer surface. The past-1 will then bond and form the CARD domain, and then form an apoptosome (i.e. a holoenzyme, combination of several proteins). This complex is a protease which cuts or degrades other proteins. Apoptosome will activate procaspase-9 to become caspase-9 (the first _caspase_ activated by cytochrome c release), then caspase-9 will activate procaspase-3 to become caspase-3. This large amount of caspase-3 will cut the cytoskeleton, forming an apoptotic body, phagocytosis, and ends with the amniotic cells' apoptosis [25].

Numerous studies have revealed that PROM occurs before contractions begin due to a focal defect in an area near to the rupture site [5]. Malak and Bell in 1999 were the first to find an area of "high morphological change" on the amniotic membrane surrounding the cervix [26]. This area makes up 2-10% of the total amniotic membrane surface. A lot of studies support a different, zonal amniotic-membrane concept, especially surrounding the cervical which is significantly weaker compared with other zones due to changes in the biochemical and histological structure.

The amniotic membrane represents the most fragile part (compared with other areas) and is known as the paracervical weak zone where the highest prevalence of apoptosis is found [5]. Another study that supports this finding is Kataoka _et al.,_ (2002) that reveals the apoptotic process on the amniotic membrane is highest at the area surrounding the cervix compared with the area at the fundus in PROM vs without PROM patients [22]. This paracervical weak zone had developed before PROM occurred and had a role as an initial breakpoint [26]. The amniotic membrane at this site shows an apoptotic process with an increase of apoptotic marker cleaved-caspase-3, cleaved-caspase-9, and a decrease of Bcl-2; these proteins represent the main proteins of the intrinsic pathway [5]. The other study that supports the current study's finding is by Menon, which reveals that the intrinsic pathway has a role in PROM due to increasing proapoptotic gene expression, p53, and decreasing Bcl-2 antiapoptotic gene expression [27]. Due to this apoptosis-based factor, the amniotic membrane becomes weaker and more susceptible to

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rupture in term pregnancies [5] [7].

The paracervical weak-zone's strength to resist rupture is only 60% compared with others areas of the amniotic membrane. The paracervical weak-zone's position is at the centre above the cannabis cervical, and this zone is 10 cm in diameter [28] [29]. Another other study reveals epithelial amniotic cell apoptosis in PROM cases; this apoptosis not only caused weakness of the amniotic membrane but also became an activation factor for metalloprotein which will degrade the extracellular matrix inducing disintegration and this will increase the amniotic membrane's fragility, although the exact mechanism remains unclear [30].

A study by Suhaimi (2010), found a similar finding from an ELISA test: the p53 level was higher in PROM patients compared with those experiencing normal labour. The changes in the pro- and anti-apoptotic proteins at the paracervical area caused weakness in the integrity of the amniotic membrane structure and increased PROM risk. The increase of p53 expression-induced amniotic membrane cell apoptosis through the activation of Bax and then Bax-induced release of cytochrome c [7].

Menon *et al.*, (2014) findings reveal that 79% of amnion cells and 89% of chorion cells in preterm PROM cases express p53. The average p53 expression in the preterm PROM group was 72% on amnion cells, and 80% on chorion cells which was higher than p53 expression in without PROM term labour, but the significance level between the two groups was 0.15 ($p > 0.05$) which is not significantly different [31].

Arofah *et al.*, (2005) explain their finding that p53 expression in amniotic cells from the edge of a rupture taken from PROM patients was higher than p53 expression in without PROM patients in term pregnancy; the average p53 expression in the PROM group was $21.50 \pm 1.27\%$ compared with $5.40 \pm 2.12\%$ in the without PROM groups. A strong p53 expression from the edge of the rupture significantly correlated with PROM incidence according to statistical testing [32].

A study of amniotic membrane apoptosis in PROM cases using the IHC TUNEL method to highlight the apoptotic nuclei reveals that apoptotic activity on the amniotic membrane in PROM labour is higher than in without PROM labour [33]. A recent study explains that the proapoptotic protein signalling pathway (including p53) plays a role in the senescence process through amniotic membrane apoptosis which determines the time of labour [8].

Negara *et al.*, (2017) reveal that apoptosis is increased in PROM patients through the increase of proapoptotic protein caspase-3, apoptosis-inducing factor (AIF), and Bcl-2. Apoptosis in both the caspase-dependent pathway and the caspase-independent pathway play a role in PROM occurrence [23].

Arofah et al., (2005) study suggests that the amniotic-membrane cell apoptotic index taken from the edge of a membrane rupture site of a PROM group was higher than the without PROM groups at term pregnancy, with the apoptotic-index average of the PROM group $37.30\% \pm 9,57\%$ vs. $9.80\% \pm 3,33\%$ in the without PROM groups. Amniotic-cell apoptotic index in this study was performed using a conventional method of routine haematoxylin-eosin staining [32].

A recent study showed that that the p53 signaling pathway played a role in the senescence process through amniotic membrane apoptosis, which would determine the time of labor [8].

In summary, p53 expression and the apoptotic index of amniotic membrane cells in cases of PROM were higher than those in without PROM cases; there was a strong correlation between p53 expression and the apoptotic index with the occurrence of PROM. The strong apoptotic index was correlated with strong p53 expression in PROM cases at term pregnancy.

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