High Apoptotic Index in Amniotic Membrane of Pregnant Women is A Risk Factor for Preterm Labor

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ABSTRACT

This study aims to prove a high apoptotic index in the amniotic membrane as a risk factor for preterm Labor. Case-control study, comparing preterm and term groups (n=54) who underwent labor in Obstetrics Emergency Room, Sanglah Central General Hospital Denpasar, Bali. Apoptotic index is a method of identifying and assessing the quantity of tissue undergoing apoptosis, which in this study was the amniotic membrane tissue, calculated and stained using the TUNEL method. Data collected was subjected to further statistical tests. Normally test with Shapiro Wilk test, followed by parametric independent T-test and Mann Whitney test was performed. Cut off apoptotic index was determined using the Receiver Operating Characteristic (ROC) curve. Bivariate analysis with Chi-square test and calculation of odds ratio was performed to assess the high apoptotic index to the risk of preterm Labor. Statistical significance was obtained with p value <0.05. Based on the cut-off value of ROC, the apoptotic index was classified into high (≥37.5%) and low (<37.5%) apoptotic index. A high apoptotic index is a risk factor for preterm Labor and increases the risk 6 times greater than the group with a low apoptotic index (p=0.003; OR 5.714; 95% CI 1.764–18.507). High apoptotic index in the amniotic membrane of pregnant women is a risk factor for preterm Labor.

Keywords: apoptotic index, preterm labor.

I. INTRODUCTION

Preterm Labor occurs prematurely and is still a global problem because it is the largest contributor to perinatal mortality and neonatal morbidity in the short and long term. Various severe morbid conditions such as respiratory failure syndrome, intraventricular hemorrhage, and necrotizing enterocolitis are more common in preterm infants than in term infants. Long-term disorders such as cerebral palsy, visual impairment, and hearing loss are also more common in preterm infants [1], [2]. This can result in a low quality of human resources in the future. In addition, the care of premature babies also requires sophisticated and expensive medical technology, which will indirectly increase the economic burden on the family and the country.

Prematurity is the leading cause of neonatal and under-five mortality. Thirty-five percent of neonatal deaths worldwide are caused by preterm birth complications [3]. Preterm birth is still an epidemic worldwide; the global incidence is estimated at 15 million yearly [4]. Data from 184 countries found that the incidence of preterm Labor ranged from 5% to 18% of deliveries [5]. Indonesia is one of 10 countries with high rates of prematurity, with a total of 675,700 births or 18% of deliveries [5]. Indonesia is one of 10 countries with high rates of prematurity, with a total of 675,700 births or 18% of deliveries [5].
Hospital study reported a 9.4% incidence of preterm birth, or 138 cases out of 1468 live births. Data from Sanglah Hospital in 2015 reported that the number of preterm deliveries increased by 285 cases from 1198 live births or 23.7%, and within 2 years, from January 1, 2017–December 31, 2018, cases of preterm deliveries were found in the Obstetrics Emergency Room (ER). Sanglah Hospital Denpasar increased by 555 cases (29.5%) of all deliveries [6], [7].

Preterm Labor is a pathological process resulting from multifactorial causes. The risk factors associated with preterm Labor can be broadly divided into 3 main sources: maternal, fetal, and umbilical cord. Judging from the pathophysiology, currently, there are four main mechanisms of preterm Labor, including infection and inflammation, uterine mechanical stretching, decidual bleeding, and premature activation of the maternal-fetal hypothalamic axis or the maternal-fetal hypothalamic-pituitary axis (HPA).

One of the main mechanisms of preterm Labor is infection and inflammation through the apoptotic pathway. Infections and endotoxins can stimulate many pro-apoptotic factors derived from the amniotic membrane during preterm Labor. Physiologically there will be changes in the biochemical and mechanical properties of the amniotic membrane where amniotic cells undergo apoptosis at term until they enter the delivery phase. The nature and function of the fetal membranes maintain and ensure the nutritional needs of the fetus during pregnancy. So, the maintenance of the integrity of the amniotic membrane during pregnancy is necessary for fetal development. In apoptosis, there was an increase in the breakdown of ADP-ribose polymerase I, a marker of apoptosis, a decrease in inhibitor of tissue metalloproteinase 3 (TIMP-3), and changes in the histology of cell apoptosis. Cell death or apoptosis is seen in the amnion and chorion in term and preterm ruptured membranes [8], [9].

The role played by MMPs is critical in apoptosis in ruptured fetal membranes. The strength of the amnion and chorion is largely due to collagen. Collagen I, III, IV, V, and VI are present in various layers of the amniochorion. The main forces in the amnion are derived from collagen I (extensively seen in the compact layer and adjacent mesoderm) and collagen IV, which are major components of the basement membrane and from the bundles connecting the mesenchymal and epithelial layers, but the physiological mechanisms that initiate MMP activation and apoptosis in membranes is still not widely known. Evidence that supports that the process of apoptosis plays a role in the rupture of the amniotic membrane is the finding of 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 [10].

The process of apoptosis can occur under abnormal conditions that are associated with a cause of preterm Labor. Conditions of hypoxia, cell injury due to infection, oxidative stress, and radiation will activate the levels of pro-inflammatory cytokines, thereby accelerating the apoptotic process that causes rupture of the membranes, which will trigger uterine contractions at least 10% in term Labor and nearly 30% in preterm Labor ending in delivery. [11], [12], [13].

The apoptotic index is the number of cells undergoing apoptosis. Research shows that the membranes in delivery with premature rupture have a higher apoptotic index than those without premature rupture of membranes. Using the TUNEL method, the identification of apoptotic cells in the amniotic epithelium, chorion cells, and decidua parietal showed chromatin condensation in the cell nucleus [14], [15].

The relationship between the process of apoptosis in the amniotic membrane and its impact on delivery has been widely reported, but the results of this study are still controversial. The apoptotic index in spontaneous or vaginal delivery was higher than that of cesarean section. This process occurs at term gestational age, which is thought to function in initiating delivery [8]. A previous study found a higher apoptotic index in the 23–30 weeks group compared to the 31–36 weeks group in the amniotic epithelial cell layer using the TUNEL method. This condition is thought to be due to the sample studied being pregnant with complications such as chorioamnionitis and history of ruptured membranes [16]. The results of this study are supported by several other studies, which reveal that a higher apoptotic index in amniotic cells is one of the causes of preterm Labor [17].

A different opinion was reported by Saglam, which stated that the increase in apoptotic processes that occurred in preterm labor was not significant. This condition was found in the preterm Labor group without a history of ruptured membranes. Although the activation of proinflammatory cytokines in this condition increased, the severity of infection that usually occurs will increase the apoptotic process [14]. Similar findings were conveyed by Fortunato, who stated that there was an incomplete apoptotic process in preterm Labor due to an increase in anti-apoptotic factors that caused protection against the apoptotic process. In addition, pro-apoptotic cytokines were reported not to be found in preterm Labor, especially in cases of preterm Labor without a history of ruptured membranes, but the factors that initiate this condition are unknown [18].

Measurement of markers of the inflammatory process can be a method for early detection of women at high risk for preterm Labor, one of which is the apoptotic index [17], [19]. The role of apoptosis on the incidence of preterm Labor is still being explored, considering the many mechanisms and factors that influence it, so researchers are interested in conducting this study. Knowledge of these aspects is expected to help improve the management, intervention, and management of preterm labor.

II. DISCUSSION

This study was conducted with a case-control study design on 54 samples divided into 27 groups of mothers with preterm labor and 27 groups of mothers with term labor. These maternity mothers were patients who visited the Maternity Room of the Obstetrics and Gynecology Emergency Room at Sanglah General Hospital in Denpasar from March 2021 to August 2021. This study has received ethical approval from the Udayana Medical Faculty Research Ethics Commission.

A. Characteristics of the Research Sample

The variables were studied descriptively on the sample characteristics of the two groups: maternal age, parity, and BMI. In presenting the data from the numerical variables, analytical methods were used to determine the distribution of
Based on the overall data from the case and control groups that met the inclusion and exclusion criteria, an analysis test was carried out to see the data distribution in this study. The sample characteristic data's normality test was carried out using the Shapiro–Wilk test (sample < 50 each arm), interquartile range (IQR) in each group.

This study included 54 samples that met the inclusion and exclusion criteria, consisting of 27 samples as cases and 27 others as controls. In this study, the median age of the mother in the preterm labor group was 27 (15–45) years, and the median age in the term labor group was 26 (21–39) years, with a P-value = 0.477. This means there is no statistically significant age difference between the preterm and term labor groups. Previous studies stated no difference in maternal age between the preterm labor groups on the indication, spontaneous, or term preterm labor [20]. The results of this study were also presented by Daglar et al. [21] that there is no difference in the mean age of the mother between groups with term labor, the threat of preterm labor, and preterm labor. The mean age of mothers in the preterm labor group was higher than that of mothers in the threatened preterm labor group who reached term (26.4 years versus 24.4 years) [21].

Different from the study conducted by Fuchs et al. [22], who found a relationship between maternal age and the incidence of preterm labor. Data from 165,282 deliveries at 32 hospitals in Canada showed that maternal age of 40 years was associated with an increased risk of preterm labor, with the lowest risk of preterm labor being found in women in the 30–34-year age group, increasing the risk of events by 1.2 times and 1, respectively..15 times [22].

In this study, the median parity in the preterm labor group was 0 (0–4), and the median parity in the term labor group was 1.00 (0–3) with a P-value = 0.375. This shows that there is no statistically significant difference in parity between the two groups. Based on previous studies, the age group of mothers with preterm labor was dominated by the age group of 20–34 years (81.6%), while the parity group was dominated by the primigravida group (50.9%) [23]. Another study also showed similar results, with the predominant age being 21–30 years old, but neither age nor parity had a statistically significant effect on the incidence of preterm labor [24].

Different from the study conducted by Koullali et al. [25], who assessed the association between parity and the incidence of preterm labor in 30,237 pregnancies and found an increased risk of preterm labor in nulliparous women (OR 1.95; 95% CI 1.89–2.00) compared to women in the second pregnancy. The risk is significantly increased due to delays in antenatal care and maternal illness [25].

In this study, the median BMI in the preterm labor group was 23.2 (16.65–31.96) kg/m², and the median BMI in the term labor group was 22.04 (17.97–34.24) kg/m² with a P-value = 0.665. It also shows no statistically significant difference in BMI between the two groups. A study conducted by Slack et al. found a relationship between maternal obesity and preterm labor, with a higher risk of occurrence in mothers with grade III obesity (OR 2.80; 95% CI 1.31–5.98) [26]. Similar results were presented in the study of Cnattingius et al. in Sweden, with a total sample of 1,857,822 [27]. There were 5.03% of preterm deliveries, with the incidence of labor increasing along with the increase in BMI, especially in BMI 25–29 (overweight) and 30–34 (obese). In other words, maternal BMI is correlated with the incidence of preterm labor [27]. A different opinion was expressed by Hendler et al. (2005); in women with BMI <19 kg/m², it was found that 16.6% had a preterm labor, at BMI 19.1–24.9 kg/m² as much as 11.3%, at BMI >25–29.9 kg./m² as much as 8.1% and BMI 30–34.9 kg/m² as much as 7.1%. Compared with normal BMI women, preobese and grade I obesity have a 15% lower risk of preterm labor. The reported significantly lower prevalence of short cervix found to be obese compared to normal or thin BMI women may explain the reduced risk of preterm labor in obesity [28].

There were no statistically significant differences in the characteristics of maternal age, parity, and BMI in the two sample groups, so it can be said that the sample characteristics did not influence the analysis results of increasing the apoptotic index.

The analytical method was carried out to determine the homogeneity of the data in this study. Statistically, the test of variance or homogeneity of the data was carried out using Levene's test, with a P-value of > 0.05, which means the data variance is homogeneous. After the test, it was found that each group of variants of the characteristics of the research sample had homogeneous data. The next step is to determine the significance of the difference in the median obtained and the results of the distribution of data that are not normally distributed using the Mann–Whitney test.

In the case group, the median age of the mother was 27 years, with the lowest maternal age being 15 years and the highest being 45 years, while in the control group, the median age was 26 years, with the lowest maternal age being 21 years and the highest being 39 years. Characteristics of the sample based on parity obtained a median of parity status in the case group 0, with the lowest value of parity 0 and the highest value of parity 4, while the group obtained a median of 1, with the lowest value of parity 0 and the highest value of parity 3. In the next variable, characteristics based on BMI were obtained: the median in the case group was 23.24 kg/m², with the lowest value 16.65 kg/m² and the highest value 31.96 kg/m², while in the control group, the median was 22.43 kg/m² with the lowest and highest values 17.97 kg/m² and 34.24 kg/m², respectively. Based on the Mann–Whitney test, there was no statistically significant difference between the two groups based on maternal age, parity, and BMI (p > 0.05; p = 0.477; p = 0.375; p = 0.665; respectively).

B. Calculation of the Levels of the Apoptotic Index in the Sample Group

The apoptotic index was calculated on the membranes based on the number of research samples, as many as 54 pregnant women. Amniotic tissue was taken from the edge of the amniotic membrane, measuring 2 cm wide after delivery, and then placed in phosphate buffer saline (PBS) fixation solution. The samples were then sent to the Integrated Biomedical Laboratory, FK UNUD Denpasar, for examination of the apoptotic index using the TUNEL IHK method.

Data normality test using the analytical method using Shapiro Wilk (sample <50 each arm) showed that the

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apoptotic index data based on the birth group were not normally distributed (p<0.05), so the median (median) and interquartile range (IQR) were used to present the distribution of the data. In each group. The distribution of the apoptotic index descriptively by the labor group can be seen in Fig. 1.

It was found that the median apoptotic index in the preterm group was higher than that in the term group (48% versus 15%). In the case group, the median apoptotic index was 48%, with the lowest value of 7% and the highest value of 83%, while in the control group, the median apoptotic index was 15%, with the lowest value of 7% and the highest 61%.

The calculation of ROC was carried out in this study to determine the cut-off at the optimal point of sensitivity and specificity of the apoptotic index that could be achieved. The resulting ROC’s area under the curve (AUC) value is 0.708 (p=0.009). The cut-off point of the determined apoptotic index was 37.5%, with a sensitivity of 66.7% and a specificity of 74.1%.

In this study, apoptotic nuclei would be easier to see due to IHK TUNEL staining, where all core fragments were stained brown. Most of the apoptotic nuclei in this study based on the Odds Ratio (OR).

Based on the Chi-square test analysis results, there was a statistically significant difference (p<0.05) between the case and control groups. A high apoptotic index is a risk factor for preterm labor and increases the risk of the incidence 6 times greater than the group of patients with a low apoptotic index (p=0.003; OR 5.714; 95% CI 1.764–18.507).
progression, and apoptosis [13, 31]. Cytokine expression in the chorion, amnion, and decidua has a role in delivering signals for apoptosis. High levels of proinflammatory cytokines play a role in delivery by stimulating the degradation of the extracellular matrix in the cervix [29, 30].

The role played by MMPs is critical in apoptosis in ruptured fetal membranes. The strength of the amnion and chorion is largely due to collagen. Collagen degradation is controlled by matrix metalloproteinases which have different specificity for each type of collagen; matrix metalloproteinases can be modulated by matrix metalloproteinases (TIMPs) tissue inhibitors. The MMP/TIMP ratio in collagen determines whether the collagen will be degraded or not. MMP activation and apoptosis are often interrelated. The extracellular matrix plays a major role as a balancing factor in many tissue systems. This stability is compromised when MMP activation causes damage to the extracellular matrix and causes apoptosis [33].

Proinflammatory cytokines can trigger cervical effacement in several ways. IL-1β and TNF-α increase the production of MMP-1, MMP-3, MMP-9, and cathepsin. In addition, IL-1β suppresses the expression of tissue inhibitor of metalloproteinase (TIMP)-2, an endogenous MMP-2 inhibitor. Some proteinases can digest collagen and elastin fibers in the cervical extracellular matrix, further increasing cervical compliance. IL-1β can act on several cell types to increase the production of COX-2 and prostaglandin E2 (PGE2), potent chemicals that induce cervical dilation in women. PGE2 can stimulate delivery by increasing the production of proteinases or through an indirect pathway, namely by increasing the permeability of blood vessels to enter leukocytes [9].

Excessive apoptosis will cause cervical effacement and uterine contractions and damage the integrity of the membranes during pregnancy, which allows preterm labor to occur. In this study, a high apoptotic index was a risk factor for preterm labor and increased the risk 6 times greater than the group with a low apoptotic index (p=0.003; OR 5.714; 95% CI 1.764–18.507).

Preterm labor is not only related to mechanical and chemical factors but also includes the role of the apoptotic process, where the event is thought to occur before contractions occur, which are triggered by an increase in prostaglandins. Several previous studies have also explained that most of the incidence of preterm labor is associated with infection. The presence of morphological changes due to infection with characteristics of inflammation, connective tissue damage, and thinning of the trophoblast layer is associated with increased apoptotic processes in trophoblasts and thinning or loss of decidua [9, 34, 35].

Physiologically, the process of apoptosis increases with increasing gestational age, but in preterm pregnancy, the process occurs earlier, so this is what this study wants to prove. Apoptosis is a normal part of the development and maintenance of a multicellular organism. This cell death is a response to various stimuli, both intrinsic and extrinsic. Apoptosis occurs through two main pathways, namely the intrinsic and extrinsic pathways. The intrinsic pathway is the dominant pathway that plays a role in the process of apoptosis in the amniotic membrane at term. The intrinsic pathway is centered on the mitochondria, with the main regulator being the Bcl-2 protein family. Proteins of the Bcl-2 family can be either pro-apoptotic or antiapoptotic. The main site of action of these Bcl-2 proteins is the outer mitochondrial membrane. Where in this membrane are stored apoptogenic factors (cytochrome c, Smac, Diablo, AIF, and endonuclease G), which, when released, will activate the executor of apoptosis, namely caspase. Antiapoptotic proteins of the Bcl-2 family inhibit the release of this apoptogenic factor, whereas members of the pro-apoptotic group trigger the release [14].

Research by Ingle et al. [36] stated that prevention of increased apoptosis through caspase 3 activation is important in inhibiting uterine myocyte contractility during pregnancy. This study reported that the uterus during pregnancy produces an unfolded protein response to inhibit caspase 3 activity which effectively inhibits uterine contractility, thereby preventing the onset of preterm labor [36].

The apoptotic index is a method for identifying and assessing the quantity of tissue undergoing apoptosis, which in this study is the amniotic membrane tissue. The apoptotic index was calculated based on the number of cells undergoing apoptosis and apoptotic bodies detected using Terminal deoxynucleotidyl transferase-mediated deoxy Uridine Triphosphate Biotin Nick End-Labeling (TUNEL) [14], [15].

Based on the ROC cut-off value, the apoptotic index in this study was classified into high (≥37.5%) and low (<37.5%) apoptotic index. The median apoptotic index in the preterm labor group was 48%, with an interquartile range of 43%, while in the preterm labor group, the median apoptotic index was 15%, with an interquartile range of 31%. Most mothers with preterm labor (66.7%) had a high level of the apoptotic index, and 25.9% of the mothers with term labor had a high level of the apoptotic index. Based on the results of the Chi-square test analysis, it was found that there was a statistically significant difference (p<0.05) in the apoptotic index in the preterm and term labor groups.

Gotsch et al. [37] said that the concentration of caspase-1 in amniotic fluid taken from amniocentesis varies according to gestational age. Women with spontaneous delivery at term had a higher median caspase-1 than women at term without delivery. Where women who underwent preterm labor with intraamniotic infection had a higher median caspase-1 than those without intra-amniotic infection, this suggests that caspase-1 participates in the mechanism leading to spontaneous delivery at term, as well as in preterm labor that results in spontaneous labor, associated with infection/inflammation [37]. This is similar to a study by Murtha et al. [38], who examined the presence of apoptosis in the membranes using the TUNEL method. The results of this study concluded that the apoptotic activity in term labor in the membranes with intra-amniotic infection was higher than in the membranes without intra-amniotic infection (11.2% vs. 5%) [38].

It was also reported that the increase in prostaglandins with the induction of apoptosis in the amniotic epithelium and mesenchyme by apoptotic agents non-physiological (actinomycin D, cycloheximide, staurosporine) and physiological (ceramide, lactosylceramide, metabolite PGJ2). Prostaglandins also induce transcription and activate MMPs in most tissues. The apoptotic process can weaken the
amniotic membrane by eliminating fibroblast cells, which function to build new collagen and simultaneously activate an enzyme system that breaks down existing collagen. MMP activation further increases apoptosis, providing feedback in the form of increasing MMP activation. MMP activation and apoptosis have been shown to work synergistically to cause the rupture of membranes [33].

Research by Menon et al. [39]. In conclusion, Menon's research showed that infection could induce oxidative stress and apoptosis in the amniotic membrane. In this experiment, Menon used materials from amniotic membranes (amnion and chorion) obtained from term pregnant women after cesarean section, which was then divided into three groups. The first group was given a smoke extract from cigarettes, the second group was given lipopolysaccharide (LPS) from Escherichia coli, and the third group was given both. After 24 hours of stimulation, the infection markers were examined, namely cytokines, TNF, and MMP, as well as apoptotic markers, namely p53, caspase-3, and cPARP-1. It was concluded that infection (in this case played by LPS) mediates the formation of tolls like receptor (TLR) and tissue receptor of myeloid cells (TREM), which then triggers the inflammatory process (increased cytokines / MMP), which causes degradation of the extracellular matrix and the occurrence of preterm labor. Prostaglandin intermediates. The inflammatory response, in this case, was inhibited by cleavage of PARP-1 and the direct inhibitory effect of apoptosis on NF-KB. Furthermore, the initiation of preterm labor is isoprostane mediated [39].

Several other studies with similar results were found in various places. Research conducted by Harirah et al. [8] at the Department of Obstetrics and Gynecology at the University of Texas Medical Branch explained the relationship between the apoptotic index of the amniotic membrane based on the mode of delivery. The sample in this study amounted to 16 samples, namely 8 samples of the amniotic membranes from cesarean section and spontaneous delivery. Amniotic tissue was taken from the middle of the membrane away from the edge of the amniotic membrane tear measuring 3 cm wide after delivery, then placed in phosphate buffer saline (PBS) fixation solution. The TUNEL CPI examination also determined the apoptosis index in this study as in this study. The results of this study explained that an increase in the apoptotic index in the chorion trophoblast from ruptured distal amniotic membranes after vaginal delivery was 3 times higher than that of cesarean section (11.57±3.98% and 4.05±2.4%, respectively). -respectively, p=0.012). The choriodecidual layer after vaginal delivery showed higher expression of active proapoptotic caspase 3 and lower expression of anti-apoptotic Bcl-2 than in cesarean section [8].

Another study was conducted by Runic et al. in New York [15]. This study aimed to determine the presence or absence of Fas expression in amniotic membrane cells undergoing apoptosis based on morphological and biochemical parameters. Based on biochemical data, the study reported that apoptosis is a physiological process in the amniotic membrane that usually occurs in the third trimester of pregnancy. Samples in this study were the amniotic membranes taken from 17 preterm cases (<37 weeks) and 21 term cases (≥37 weeks). Samples from term cases were taken from women who had a spontaneous vaginal delivery without complications or cesarean section with or without delivery. Meanwhile, samples from preterm cases were also taken from patients with complications of pre-eclampsia, diabetes, chorioamnionitis, and premature rupture of membranes. The results of this study stated that the membranes of preterm pregnant women showed a higher apoptotic index than the membranes of term labor with intact membranes. The apoptotic index in the amnion, chorion, and decidua cell layers was statistically significant in the 37–42 weeks gestational age group compared to 23–30 weeks and 31–36 weeks (p<0.01). The apoptotic index in amniotic epithelial cells was statistically increased (p<0.05) in the 23–30 weeks gestational age group compared to the 31–36 weeks gestational age group, so it is believed that the apoptotic process plays an important role in regulating the amnion, chorion, trophoblast, and epithelial cells. Decidua. The results of this study also stated that Fas expression plays an important role in mediating the process of apoptosis.

III. CONCLUSION

The study results concluded that a high apoptotic index in the amniotic membranes of pregnant women is a risk factor for preterm labor.

CONFLICT OF INTEREST

Authors declare that they do not have any conflict of interest.

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