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High Expression of Caspase-9 and Low BcL-2 on Amnion Cells as the Risk Factor for Premature Rupture of Membranes

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Abstract: ***Premature rupture of membranes (PROM) is an important problem in obstetrics that can increase perinatal morbidity and mortality as well as infection in pregnant women. The occurrence of PROM is caused by various risk factors and not yet fully known. The pathophysiology is said to be related to apoptosis process in amniotic cells. It is known that there are different pathways in the mechanism of the PROM which was related to the expression of caspase-9 and BcL-2. The aim of this study was to determine the role of caspase-9 and BcL-2 expression as the risk factors for PROM.A case control study was conducted with primary data collection in the form of amniotic membrane layer of amnion taken from the placenta after delivery in the emergency room of Sanglah Hospital Denpasar, Bali as many as 40 samples. The expression of caspase-9 and BcL-2 was examined at the Anatomical Pathology Laboratory of Sanglah Hospital Denpasar. The result of this study found there was a statistically significant difference (p = 0.000) where patients with high Caspase-9 expression had a 51 times chance of experiencing PROM compared with patients with low Caspase-9 expression. The group of patients with low Bcl-2 expression had 171 times the likelihood of developing PROM compared to the group of patients with high Bcl-2 expression (p = 0.000). As the conclusion, a high Caspase-9 expression and low BcL-2 expression are the risk factors for PROM in term pregnancy.***

Keywords: Caspase-9 expression, BcL-2 expression, amniotic cells, premature rupture of membranes

1. Introduction

Premature rupture of membranes (PROM) is an important problem in obstetrics due to complication of preterm delivery and the occurrence of chorioamnionitis infection to sepsis, which increases perinatal morbidity and mortality and causes infection in pregnant women. The mechanism of PROM at term gestation is a physiological event in the majority of cases. Risk factors for PROM are reproductive tract infections, intra-amniotic infections, abruption, trauma and apoptosis of the amniotic membrane also can be the risk factor in little proportion of PROM cases at term gestation. The incidence of PROM occurs in 10-20% of all pregnancies, 80% of them occur at term gestation. In term pregnancy, PROM has an incidence of 6-19%, whereas in preterm pregnancies it is 2% of all pregnancies [1].

Complications that occur in infants are intrauterine infection, cord compression, respiratory distress syndrome (RDS), necrotizing enterocolitis, intraventricular bleeding and neonatal sepsis. Risk factors for PROM include female reproductive tract infections (bacterial vaginosis, trichomoniasis, gonorrhea, chlamydia, and subclinical chorioamnionitis). Chorioamnionitis is found in 9% of pregnancies with PROM, where the risk increases to 24% if the rupture occurs more than 24 hours [2]. Studies have linked the incidence of amniotic membrane rupture with biochemical processes in the amniotic membrane [3].

The causes of premature rupture of membranes in pregnancy are not fully known, but the underlying pathophysiology is influenced by various factors and it is complex. The main mechanism of premature rupture of membranes is damage to the integrity of the amniotic membrane (chorioamniotic), increased collagenolytic activity, and decreased amniotic membrane collagen biosynthesis. In addition, there are risk factors for preterm pregnancy with premature rupture of membranes and low birth weight such as infection or inflammation of the choriodecidua, intrauterine infection, bacterial vaginosis, smoking, history of preterm delivery, history of preterm pregnancy with premature rupture of membranes, uterine over distention, cervical incompetence, amniocentesis, and vitamin C deficiency [4].

In preterm pregnancy, amniotic cells in PROM contain many apoptotic cells in the area adjacent to the rupture site, whereas in other locations, there are fewer apoptotic cells found. Thus, it has been suggested that another mechanism of premature rupture of membranes is the death of apoptotic cells in the fetal membrane [5].

Several studies have concluded that there are other pathways that cause rupture of the membranes before term gestation that are different from the mechanism caused by infection. This pathway is related to the high level of caspase-9 expression and low level of BcL-2 expression, it is believed that it can cause PROM. However, until now the measurement of caspase-9 expression as a trigger for PROM has not been done much so that we are interested in conducting research on this topic to determine the role of caspase-9 and BcL-2 expression as the risk factors for PROM.

2. Literature Survey

Premature Rupture of Membrane (PROM)
PROM is defined as the spontaneous rupture of the membranes before delivery and within one hour or more are not followed by early signs of labor or a wait of 2 hours is not followed by signs of labor [6].The prevalence of PROM worldwide ranges from 3-4.5%, whereas the incidence of PROM occurs in 10-20% of all pregnancies, PROMin term gestation can cause complications in 8-10% of pregnancies, of which 2-3% occur before 37 weeks of gestation [7]. At Sanglah Hospital Denpasar during 2015, there were 1450 cases of births, 212 cases of labor with PROM were found (14.62%). [8].

Amniotic Membrane Structure
The amniotic membrane consists of the amniotic layer and the chorion layer, does not contain blood vessels and nerves, so that its nutritional needs are supplied through the amniotic fluid. The chorion layer is thicker and more cellular but the amniotic layer is stiffer and stronger because it has greater tensile strength [9].

Maintenance of the tensile strength of the amniotic membrane requires a balance between the synthesis and degradation of the components of the extracellular matrix. Changes in the composition of the extracellular matrix and amniotic membranes are generally caused by an enzyme that degrades the matrix, namely matrix metalloproteinase (MMP) and is inhibited by Tissue inhibitor of matrix metalloproteinase (TIMPs). The ratio of MMP/TIMP to collagen determines whether the collagen will go through degradation or not [10].

Mechanism and Risk Factor for PROM
The area near the site of the membrane rupture has been described as a confined zone of extreme morphological changes, characterized by increased collagen tissue disruption seen in the dense, fibroblast, and spongy layers. In particular, there is thickening of the connective tissue components of the amniotic membrane, thinning of the cytotrophoblast and decidua layers, and interference between the amnion and the chorion. There is a decrease in the density of collagen I, III, and V in this zone. This zone is near the cervix before the onset of labor, implying that these changes precede the onset of labor. Observations by Bell and Malak show that changes that occur in the morphological zone are wider in premature rupture of membranes. These zones appear before the membrane ruptures and represents the starting point of rupture [11]. Some risk factors of PROM are nutrient deficiency, collagen degradation, infection, hormone, mechanical stressing, apoptosis, BcL-2 and caspase-9.

Nutrient deficiency
Vitamin C deficiency will cause the collagen structure to be not well formed, weak and easily destroyed [12]. Smoking has been associated with decreased serum ascorbic acid concentrations. Calcium in tobacco has been found to increase the iron-binding protein content of metallothionein in trophoblasts[13].

Collagen Degradation
Collagen degradation is mediated primarily by matrix metalloproteinase (MMP), which is inhibited by specific tissue inhibitors and other protease inhibitors (TIMP-1) [14]. In the human amnion and chorion, MMP-9 activity increases and TIMP-1 concentration drops dramatically at delivery [15].

Infection
Bacterial infection and inflammatory responses stimulate the production of prostaglandins by the amniotic membrane, it causes irritability of the uterus and collagen degradation in the membranes of the amniotic membrane. Prostaglandin E2 is known to interfere with collagen synthesis in the amniotic membrane and increase the activity of MMP-1 and MMP-3 [9].

Hormone
Progesterone and estradiol reduce the concentration of MMP-1 and MMP-3 and increase the concentration of TIMP in rabbit cervical fibroblasts, while relaxin hormone increases the expression of MMP-3 and MMP-9 in the amniotic membrane [16].

Mechanical Stressing
Mechanical stretching of the amniotic membrane (polyhydramnios) promotes the production of several amniotic factors, including prostaglandin E2, Interleukin-8 and MMP-1 activity in the amniotic membrane. PGE2 increases uterine irritability, decreases amniotic membrane collagen synthesis, and increases MMP-1 and MMP-3 production by human fibroblasts [13].

Apoptosis
Apoptosis activity is regulated by proapoptotic protein. In normal amniotic membranes, the expression of BcL-2 has no effect on caspase-3, although indeed in the last weeks of pregnancy there will be an increase in caspase-3 [17]. The expression of caspase-3 on the amniotic membrane is a risk factor for PROM, especially when expressed prematurely. Caspase-3 is one of the most important proapoptotic protein molecules in initiating apoptosis, especially in the amniotic membrane, through the intrinsic pathway [18].

BcL-2
The BcL-2 protein family is a regulator of apoptosis and also has several important functions. The protein family found in genes is located at the intersection between chromosome 18 and chromosome 14 (t14, 18). These chromosomes can generally be translocated which can lead to several disorders associated with apoptosis. Unlike other oncogenes, BcL-2 has a function to increase the resistance of cells and does not function to increase cell proliferation. Therefore, the molecular role of BcL-2 in cells is to prevent cell death, not to increase cell mitosis [19].

Caspase-9
Caspase is a collection of proteolytic enzymes that have a role in inflammation and cell death. Caspase activation in apoptosis is mediated by two main pathways: mitochondria or

Bcl-2-regulated (intrinsic) and death receptor (extrinsic) pathways. The intrinsic pathway is activated when there is a response in the form of cellular stress caused by cell damage (by DNA damage, cytotoxic drugs, etc.) and is regulated by Bcl-2 [20].

3. Methods

A case control study was conducted at emergency room and Obstetrics and Gynecology Polyclinic Sanglah Hospital Denpasar fromOctober-December 2019.A consecutive sampling was conducted with inclusion criteria are pregnant women with gestational age of 37-42 weeks, single live fetus and there were no signs of mother's infection. The amniotic membrane of each patient who had delivery is taken from 20 cases of normal delivery and 20 cases of term PROM delivery. The amniotic membrane samples were examined at the Anatomical Pathology Laboratory of Sanglah Hospital Denpasar to evaluate the expression of caspase-9 and BcL-2. Normality test conducted by Shapiro-Wilk Test and homogeneity test with Levene's T-test. The difference in the expression data of the two groups of caspase-9 and BcL-2 and risk expression were tested by T-independent and Chi Square test respectively.

4. Result and Discussion

Table 1 show the characteristic of samples, there were no difference in age, parity, gestational age,and BMI to PROM incidence between the two groups (p-value > 0.05). The same result was found at other studies [21, 22, 18].But different result stated by Maryuni et al (2017) that explain that the risk factors for PROM including age, parity and education of the mother.

Table 1: Characteristic of Samples

Risk Factor	*Case (n=20)*		*Control (n=20)*		*p*
	Mean	*SD*	*Mean*	*SD*	
Age	25.90	5.83	29.20	6.31	0.094
Parity	0.65	0.81	1.00	0.97	0.225
Gestational Age	38.75	1.65	39.10	1.02	0.425
BMI	26.61	5.80	24.43	4.87	0.206
**BMI : Body Mass Index*					

Table 2. Caspase-9 Expression and PROM, show that the high expression of Caspase-9 has a 51 times greater risk of causing PROM compared to low expression of Caspase-9. Caspase-9 expression poses at least a 7.6 times risk and a 343.7 times greater risk of PROM.

Table 2: Caspase-9 Expression and PROM

		Groups		RO	CI 95%	p
		Case	*Control*			
Caspase-9	High	18	3	51.0	7.6-343.7	0.000
	Low	2	17			

This is consistent with the study conducted by Negara et al (2017) that the expression of Caspase-9 is also a risk factor for premature rupture of membranes in term pregnancy. It was explained that the involvement of Caspase-9, which is a proteolytic enzyme that has a role in inflammation and cell death (apoptosis) [23].

It is known that one of the risk factors for premature rupture of membranes is the process of apoptosis. This is related to the caspase-dependent pathways that are mediated by p53. P53 activates Bax and inhibits BcL-2. Bax will decrease the permeability of the mitochondrial membrane which is caused by the weakening of cytochrome-C and affects Ca^{2+} levels by the formation of Bax tissue with several types of Bax. As a result of this mechanism, Ca^{2+} activates the transfer of cytochrome-C from the mitochondria to the cytoplasm. Cytochrome-C in the cytoplasm will bind to apoptosis-activating factor (apaf-1), which is a protein bordering or surrounded by BcL-2 on the outer layer of the mitochondrial surface [24, 25].

This results in the formation of an apoptosome such as holoenzymes, a combination of several proteins. This complex causes the degradation of other proteins. This apoptosome will activate procaspase-9 to become caspase-9[24, 25].

Caspase-9 is the first caspase to be activated due to the release of cytochrome-C), then caspase-9 will activate procaspase-3 to become caspase-3. So that it will form apoptotic bodies, phagocytosis and finally the apoptosis of amniotic cells. Based on the theory above, it can be concluded that caspase-9 is a caspase initiator that activates caspase-3, which is a key factor in the occurrence of the apotosis process which can activate procaspase-3 [23].

However, it is different from the study conducted by Kumagai et al (2001) that there is no difference regarding the caspase-9 activity of amniotic samples at 16-27 weeks of gestation and 28-39 weeks [17]. This condition is based on the theory that caspase-9 is the initiator of caspase, where the process takes time to activate the caspase executioner, causing damage to structural proteins and activation of other enzymes [26].

Table 3: BcL-2 Expression and PROM

		Groups		RO	CI 95%	p
		Case	Control			
BcL-2	Low	18	1	171.0	14.2-2053.2	0.000
	High	2	19			

Table 3 BcL-2 Expression and PROM, show that the high expression of BcL-2 has a 171 times greater risk of causing PROM at term gestation than low expression of BcL-2.BcL-2 expression poses at least 14.2 times the risk and 2053.2 times the greatest risk of PROM.

This is also in accordance with the study by Negara et al (2018) that the expression of B-cell lymphoma-2 is a risk factor for premature rupture of membranes. In this study, it was found that the lower the expression of BcL-2 can increase 10.39 times risk of the PROM. (OR = 10.39; 95% CI = 2.73-39.56; p = 0.001)[25].

BcL-2 plays a role as an anti-apoptotic protein. The role of BcL-2 as a mitochondrial membrane protector to prevent the release of cytochrome C and AIF. In the mitochondrial membrane, the BcL-2 protein is also involved in regulating the redistribution of AIF in the mitochondrial nucleus [17].

The release of cytochrome C from mitochondria can be triggered by various stress signals originating from the inside of the cell or following caspase activation stimulated by surface receptor ligands. The integrity of the outer membrane and release of cytochrome C from mitochondria is regulated by the protein from BcL-2, which consists of anti-apoptotic factors such as BcL-2 and extra-large B-cell lymphoma (BcL-XL), as well as pro-apoptotic proteins, like Bax and Bak. These proteins can heterodimerize with each other and interact with mitochondria, where they play a key role in determining whether cells will live or die. In addition, the BcL-2 family also links both extrinsic and intrinsic pathways. The intrinsic pathway is centered in mitochondria, with BcL-2 as the main regulator [25].

Contradictory studies have found that BcL-2 is not expressed in human amniotic epithelial cells at any period during pregnancy. These findings support a recent study of apoptosis in fetal membranes at term showing that fetal membranes fail to exhibit significant immunoreactivity for BcL-2 but exhibit strong immunoreactivity for Bax. Thus, apoptosis of the amniotic epithelium at term can be induced by BcL-2 regulation which is involved in fragility and rupture of human fetal membranes at term [27, 18].

In the study sample there were also 2 cases that had high BcL-2 but PROM occurred, this may be due to the effect of progesterone in inhibiting apoptosis in amniotic fluid through suppression of the expression of proapoptotic protein, BID and inhibiting TNF-alpha-induced caspase-3 activity. Research by Wang et al (2018) states that progesterone supplementation can prevent the occurrence of PROM in a high-risk female population [28].

5. Conclusion

As the conclusion, high expression of caspase-9 and low expression of BcL-2 in amniotic cells increases the risk of premature rupture of membranes.

6. Future Scope

In the future studies it is expected to estimate confounding factors, also further investigate the role of prostaglandins. It is related with the result that the high BcL-2 expression is not associated with the incidence of PROM because there is an effect of progesterone that inhibits the membrane apoptosis process.

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