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Expression and Clinical Significance of NOD-Like Receptor Protein 3 (NLRP3) and Caspase-1 in Fetal Membrane and Placental Tissues of Patients with Premature Rupture of Membrane

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: In this study, we aimed to investigate the expression of NOD-like receptor protein 3 (NLRP3) and caspase-1 in fetal membrane and placental tissues of patients with premature rupture of membrane (PROM), and to explore their role in PROM.

Material/Methods: Ninety women participated in this study: a control group of 30 healthy pregnant women, 30 with PPROM, and 30 with TPROM. Immunohistochemistry streptavidin-peroxidase (SP) assay was used to detect the protein expression of NLRP3 and caspase-1 in the fetal membrane and placental tissues. RT-PCR was used to detect the mRNA expression of NLRP3 and caspase-1 in fetal membrane and placental tissues.

Results: The results of SP showed that NLRP3 and caspase-1 were mainly expressed in the cytoplasm of epithelial cells, mesenchymal cells, and trophoblast cells in fetal membranes, and the cytoplasm of placental syncytiotrophoblasts and vascular endothelial cells in placental tissues. The expression of NLRP3 and caspase-1 in the TPROM group was significantly higher than that in the PPROM group and control group ($p<0.05$), and there was a significant difference between the PPROM group and the control group. The results of RT-PCR showed that the mRNA expression level of NLRP3 and caspase-1 in the TPROM group was significantly higher than that in the PPROM group and control group ($p<0.05$), and the expression of NLRP3 mRNA and caspase-1 mRNA in the PPROM group was significantly different from that in the control group ($p>0.05$).

Conclusions: The increased expression of NLRP3 and caspase-1 in fetal membrane and placental tissues may be associated with the development of PROM.

MeSH Keywords: Caspase 1 • Fetal Membranes, Premature Rupture • Placenta

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Background

The incidence of premature rupture of membranes (PROM) is 5~15% outside China and 2.7~7% within China [1]. PROM may lead to premature labor, placental abruption, chorioamnionitis, and neonatal respiratory distress syndrome, which pose a serious threat to the health and life of pregnant women and their fetuses. At present, reproductive tract infection is one of the major causes of PROM. In recent years, due to changes in beliefs, risky sexual behavior, multiple induced abortion, and multiple sexual partners, the incidence of female genital tract infection has been rising and the age of patients has been getting younger. Changes in hormone levels can also lead to increased incidence and recurrence of reproductive tract infection during pregnancy. To date, the incidence of PROM is still high, but we still lack effective prevention and treatment measures.

The NOD-like receptor (NLR) family is a kind of pattern recognition receptor located in the cell, which can recognize the invading microorganism and the dangerous signals in the body, and participate in the innate immune response. NLRP3 is a member of the NLR family. Since the concept of inflammatory bodies was proposed in 2002, NLRP3 inflammatory body has been an important research focus in recent years, and it participates in a variety of nonspecific immune responses to autoimmune and infectious diseases. Bruno et al. [2] found that NLRP3 inflammatory body is involved in the immune response of female genital vaginal candida disease. Maneta et al. [3] showed that NLRP3 inflammatory body mediated the release of IL-1β in pregnancy tissues, including placenta, fetal membranes, umbilical cord blood, and amniotic fluid. Hoang et al. [4] confirmed the high expression of NLRP3 inflammatory body in isolated fetal membranes infected by bacteria. However, whether NLRP3 inflammatory body is involved in the occurrence and development of PROM has not yet been reported.

In this study, we examined the expression of NLRP3 and caspase-1 in fetal membrane and placental tissues, and discus the relationship between the expression of NLRP3 and caspase-1 and the development of PROM, so as to find a new target for the prevention and treatment of PROM.

Material and Methods

Data collection

A total of 60 patients with PROM who underwent maternity hospitalization in Xuzhou Maternity and Child Health Care Hospital from October 2014 to May 2016 were selected in the present study. Among them, 30 cases were the preterm premature rupture of membrane patients (PPROM group; gestational age: 28 weeks ~ 37 weeks; average age: 27.63±3.79 years old); the other 30 cases were the term premature rupture of membrane patients (TPROM group; gestational age: ≥37 weeks; average age: 27.20±4.16 years old). Thirty women with healthy pregnancy in the same period were selected as the control group (gestational age: ≥37 weeks; average age: 28.00±3.88 years old). All women of the 3 groups were single-pregnancy and there was no statistically significant difference between the average age of the 3 groups (p>0. 05). None of the women in the 3 groups were pregnant with complications. This study was approved by the Ethics Committee of Xuzhou Maternity and Child Health Care Hospital. Informed consent was obtained from every patient.

Diagnostic criteria: the diagnostic criteria for PROM were from the eighth edition of "Obstetrics and Gynecology" [5].

Inclusion criteria were: all the pregnant women with PROM had genital tract infection during pregnancy, and vaginal secretions or amniotic fluid cultures were positive during the first 2 weeks of the morbidity. The 3 groups of pregnant women terminated their pregnancy by cesarean section.

Exclusion criteria: 3 groups of pregnant women do not have heart, liver, kidney and endocrine diseases, excluding multiple births, hypertension, diabetes, autoimmune disease, vascular disease, infectious disease, and other parts of the maternal infection, or fetal congenital diseases.

Methods

All the research subjects were injected with antibiotics to prevent infection within 12 h after the rupture of membranes. We took the central region of the maternal surface of placenta within 15 min after cesarean section, cut off the bottom decidual layer, and took 2 pieces of the dense chorion tissue (about 1.0×1.0×0.5 cm to avoid the calcified necrotic area). Then, the tissues were cleaned by DEPC water, put in liquid nitrogen for 10 min, and then stored at –80°C. The tissues were then used to extract total RNA. At the same time, another 2 pieces of tissue block (1.0×1.0×0.5 cm) were collected, washed with 4°C saline water, fixed in 10% neutral formaldehyde for 24 h, paraffin-embedded, and then cut into 4-μm-thick slices. These 2 tissue blocks were used for immunohistochemical analysis. The immunohistochemical SP method [6] was used to detect the expression of NLRP3 and caspase-1 in placental tissues. The positive NLRP3 and caspase-1 expression slices provided by the reagent company were used as the positive controls, and PBS was used as a negative control instead of an antibody. The semi-quantitative analysis was conducted by using the comprehensive scoring method described by li et al. [7]. Under a 400× microscope, 10 visual fields were selected, and 100 cells were counted in each field of view. Cells with brownish-yellow granules in the cytoplasm were considered as the positive cells, and

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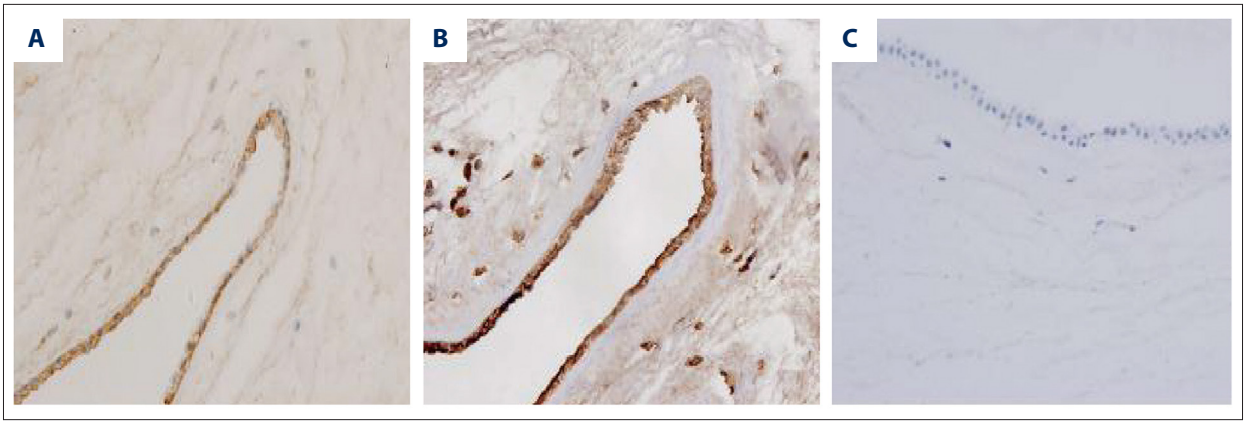

Figure 1. The expression of NLRP3 in fetal membrane tissue (DAB staining, 400×). (**A**) PPROM; (**B**) TPROM; (**C**) control group.

Table 1. Expression of NLRP3 in fetal membrane tissues.

Groups	Cases	–	+	++	+++
PPROM*	30	3 (10.00)	5 (16.67)	9 (30.00)	13 (43.33)
TPROM	30	2 (6.67)	3 (10.00)	9 (30.00)	16 (53.33)
Control*#	30	12 (40.00)	10 (33.33)	6 (20.00)	2 (6.67)

Compared with TPROM group, * P<0.05; compared with PPROM, # P<0.05.

cells with no coloration or consistent with the background color were considered negative cells. Percentage of NLRP3/caspase-1 positive cells (PP) was assessed using a 4-point scale: 0=<5% stained cells, 1=5~20% stained cells, 2=21~50% stained cells, and 3=>51% stained cells. Intensity of NLRP3/caspase-1 staining (IS) was assessed using a 4-point immunoreactivity scale: 0=colorless or consistent with background color (no staining), 1=pale yellow (weak staining intensity), 2=yellow (moderate staining intensity), and 3=brown-yellow (strong staining intensity). Overall NLRP3/caspase-1 immunoreactivity was evaluated using a resulting immunoreactivity score (IRS). IRS=PP+IS: 0~1 is negative, 2 is marked as "+", 3~4 is "+ +", and 5~6 is "+ + +".

Statistical analysis

SPSS16.0 statistical software was used for statistical analysis. Data are presented as mean ± standard deviation (SD). Comparison between 2 groups was performed by q test, comparison between multiple groups was performed by H test, and the difference was statistically significant at p<0.05.

Results

Protein expression of NLRP3 in fetal membrane tissues

As shown in Figure 1, NLRP3 is mainly located in the cytoplasm of membrane epithelial cells, mesenchymal cells, and trophoblast cells. The expression of NLRP3 in patients of the TPROM group was significantly higher than that in the PPROM group and the control group, and compared with the control group, the expression of NLRP3 in the PPROM group was significantly increased. The difference was statistically significant (p<0.05) (Table 1).

Protein expression of NLRP3 in placenta tissues.

As shown in Figure 2, NLRP3 was mainly located in the cytoplasm of the placental syncytiotrophoblast and vascular endothelial cells. The expression of NLRP3 in patients of the TPROM group was significantly higher than that in the PPROM group and the control group, and it is significantly higher in the PPROM group than that in the control group. The difference was statistically significant (p<0.05) (Table 2).

Protein expression of caspase-1 in fetal membrane tissues.

Caspase-1 is mainly located in the cytoplasm of membrane epithelial cells, mesenchymal cells, and trophoblast cells (Figure 3). The expression of caspase-1 in patients of the TPROM group was significantly higher than that in the PPROM group and the control group, and the level of caspase-1 in the PPROM group was significantly higher than that in the control group. The difference was statistically significant (p<0.05) (Table 3).

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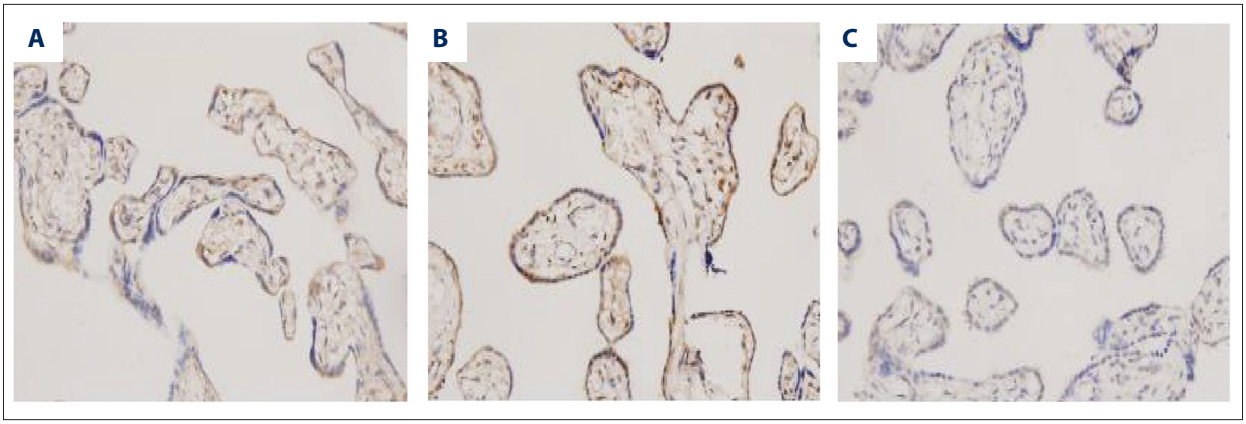

Figure 2. The expression of NLRP3 in placental tissues (DAB staining, 400×). (**A**) PPROM; (**B**) TPROM; (**C**) control group.

Table 2. Expression of NLRP3 in placenta tissues.

Groups	Cases	–	+	++	+++
PPROM*	30	3 (10.00)	4 (13.33)	8 (26.67)	15 (50.00)
TPROM	30	1 (3.33)	2 (6.67)	9 (30.00)	18 (60.00)
Control*#	30	12 (40.00)	11 (36.67)	6 (20.00)	1 (3.33)

Compared with TPROM group, * P<0.05; compared with PPROM, # P<0.05.

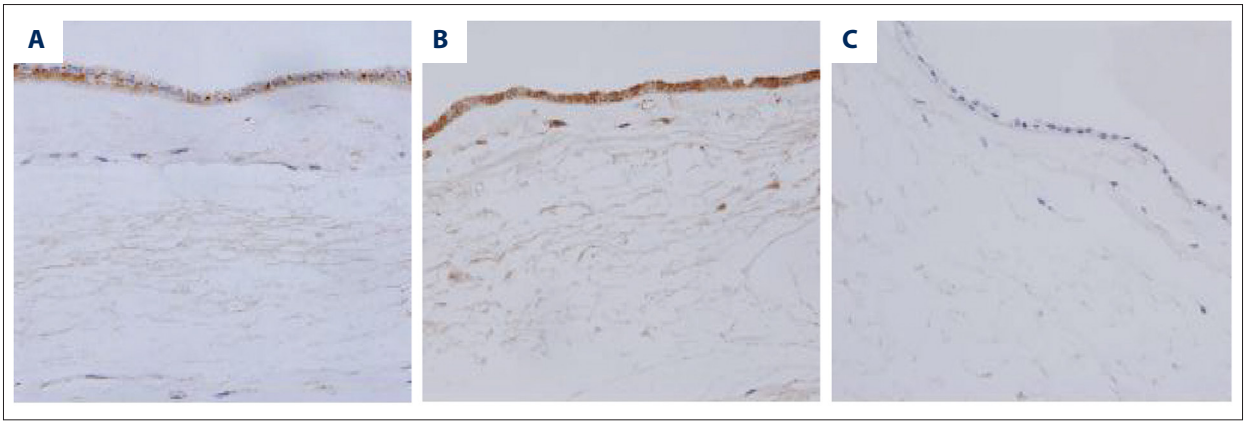

Figure 3. The expression of caspase-1 in fetal membrane tissue (DAB staining, 400×). (**A**) PPROM; (**B**) TPROM; (**C**) control group.

Table 3. Expression of caspase-1 in fetal membrane tissues.

Groups	Cases	–	+	++	+++
PPROM*	30	2 (6.67)	4 (13.33)	7 (23.33)	17 (56.67)
TPROM	30	2 (6.67)	3 (10.00)	6 (20.00)	19 (63.33)
Control*#	30	16 (53.33)	10 (33.34)	3 (10.00)	1 (3.33)

Compared with TPROM group, * P<0.05; compared with PPROM, # P<0.05.

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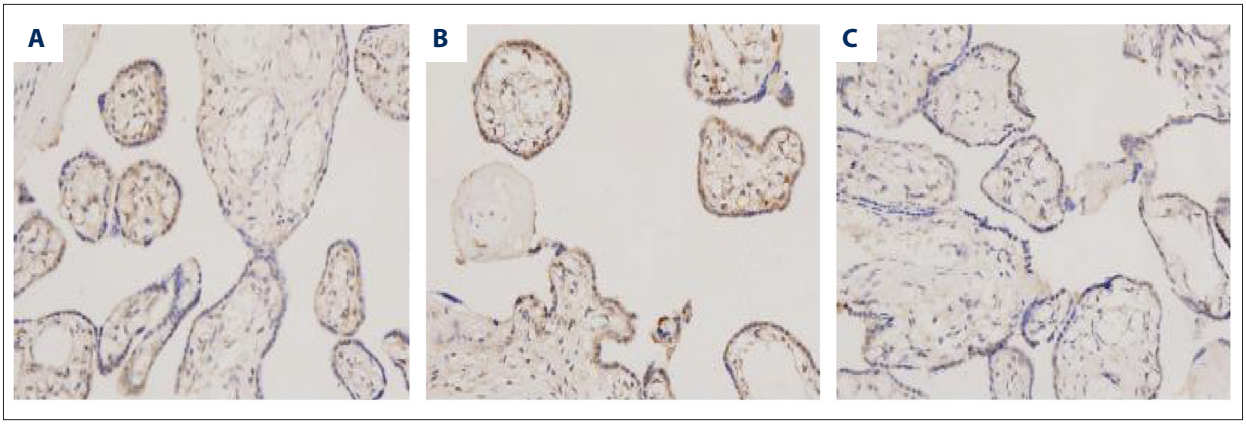

Figure 4. The expression of caspase-1 in placental tissues (DAB staining, 400×). (**A**) PPROM; (**B**) TPROM; (**C**) control group.

Table 4. Expression of caspase-1 in placenta tissues.

Groups	Cases	–	+	++	+++
PPROM*	30	2 (6.67)	3 (10.00)	8 (26.67)	17 (56.66)
TPROM	30	1 (3.33)	3 (10.00)	7 (23.33)	19 (63.34)
Control*#	30	17 (56.67)	10 (33.33)	2 (6.67)	1 (3.33)

Compared with TPROM group, * P<0.05; compared with PPROM, # P<0.05.

Protein expression of caspase-1 in placental tissues.

Caspase-1 is mainly located in the cytoplasm of placenta cells and vascular endothelial cells (Figure 4). The expression of caspase-1 in patients in the TPROM group was significantly higher than that in the PPROM group and the control group. The expression level of caspase-1 in the PPROM group was significantly higher than that in the control group. The difference was statistically significant (p<0.05) (Table 4).

The mRNA expression level of NLRP3 and caspase-1 in fetal membrane tissues

The RT-PCR product electrophoresis showed that NLRP3 and caspase-1mRNA amplification fragments were located in the vicinity of 192bp and 160bp, and both of them are expressed in the PPROM group, TPROM group, and the control group in fetal membrane tissues (Figure 5). The mRNA expression of NLRP3 and caspase-1 in the fetal membrane tissues of patients in the TPROM group was higher than that in the PPROM group and the control group (p<0.05). Moreover, the mRNA expression level of NLRP3 and caspase-1 in the PPROM group was significantly higher than that in the control group (p<0.05) (Table 5).

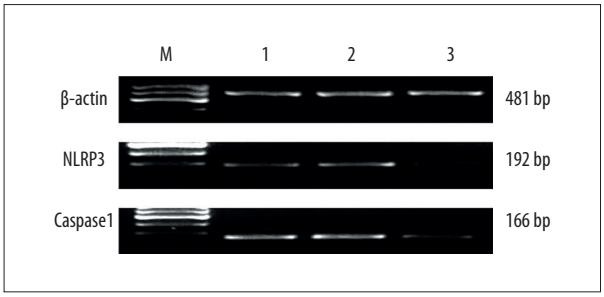

Figure 5. The expression of NLRP3 and caspase-1 mRNA in fetal membrane tissues. M – marker100~600 bp; 1 – PPROM group; 2 – TPROM group; 3 – control group.

Table 5. The mRNA expression level of NLRP3 and caspase-1 infetal membrane tissues.

Group	Cases	NLRP3/β-actin	Caspase-1/β-actin
PPROM	30	0.98±0.12*	1.01±0.06*
TPROM	30	1.14±0.13	1.08±0.06
Control	30	0.45±0.11*#	0.72±0.09*#

Compared with TPROM group, * P<0.05; compared with PPROM group, # P<0.05.

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Table 6. The mRNA expression level of NLRP3 and caspase-1 placental tissues.

Group	Cases	NLRP3/β-actin	Caspase-1/β-actin
PPROM	30	0.95±0.15*	0.93±0.14*
TPROM	30	1.09±0.12	1.13±0.12
Control	30	0.79±0.08*#	0.68±0.07*#

Compared with TPROM group, * P<0.05; compared with PPROM group, # P<0.05.

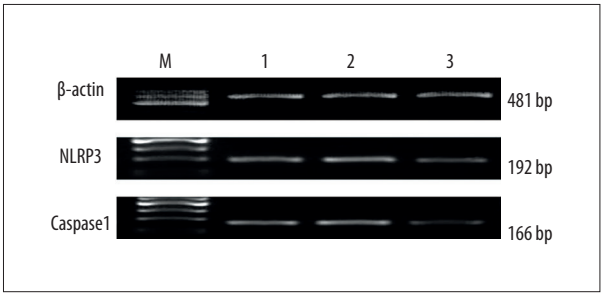

Figure 6. The expression of NLRP3 and caspase-1 mRNA in placental tissues. M – marker100~600 bp; 1 – PPROM group; 2 – TPROM group; 3 – control group.

The mRNA expression level of NLRP3 and caspase-1 in placental tissues

The RT-PCR product electrophoresis shows that NLRP3 and caspase-1 mRNA amplification fragments are located in the vicinity of 192bp and 160bp, and both of them were expressed in the PPROM group, TPROM group, and control group (Figure 6). The expression level of NLRP3 and caspase-1 mRNA in the placental tissues of patients in the TPROM group was higher than that in the PPROM group and the control group (p<0.05). The mRNA expression level of NLRP3 and caspase-1 in patients of the PPROM group was significantly higher than that in the control group (p<0.05) (Table 6).

Discussion

It is well known that infection is an important cause of PROM. The pathogen associated molecular pattern (PAMP) is recognized by the body-specific pattern recognition receptors (PRR) after the pathogen infects the body, initiating a nonspecific immune response. PRR is mainly divided into Toll-like receptors (TLR) located on the cell membrane and NLR located in the cell. NLRP3 is a member of the NLR family, which forms NLRP3 inflammatory body in the presence of apoptosis-associated speck-like protein containing a CARD (ASC) and pro-caspase-1, and adjusts the caspase-1 activation, promotes the cleavage of pro-IL-1β, pro-IL-18 and pro-IL-33 and caspase-1 dependent programmed cell death, and induces cell death under inflammatory and stress pathological conditions. NLRP3 is involved in the development of various diseases and has become an important research focus in recent years.

A study by Hoang et al. [7] found that *in vitro* culture of fetal membranes infected by pathogens shows high expression of NLRP3 inflammatory body. Pontillo et al. [8] found that NLRP3 inflammatory body is involved in the nonspecific immune response of placenta. In the present study, we found that NLRP3 and caspase-1 were mainly located in the cytoplasm of the membrane epithelial cells, mesenchymal cells, and trophoblast cells, and in the cytoplasm of placental syncytiotrophoblast and vascular endothelial cells. Compared with the normal controls, the expression of NLRP3 and caspase-1 in the fetal membrane and placental tissues was significantly increased, indicating that NLRP3 and caspase-1 might be involved in the immune response of PROM. The results of the present study are consistent with the results of previous experiments. However, our present study detected the expression of NLRP3 and caspase-1 in the fetal membrane and placental tissues of PROM patients *in vivo*, and using an *in vivo* approach is further scientific confirmation of the results of previous studies.

In addition, the experimental results showed that the expression of NLRP3 and caspase-1 in the fetal membrane and placental tissues of the patients in the PPROM group was significantly lower than that in the TPROM group, and this difference suggests that the expression of NLRP3 and caspase-1 inflammatory bodies may be different in the fetal membrane and placental tissues of patients of these 2 groups.

During pregnancy, the change of progesterone in pregnant women is on the rise; it increases slowly in the early stage of pregnancy, accelerates after 13 weeks, and reaches the peak at full term. Studies have confirmed that the progesterone receptor is expressed in the monocytes/macrophages of the amniotic membrane, chorion, and decidual tissues [9,10]. Progesterone can inhibit the release of inflammatory cytokine TNF and IL-1 in placenta, thereby inhibiting the generation of MMP-1 and MMP-3 in decidual cells and preventing TNF-mediated apoptosis [11–13]. A study by Kumar et al. [14] shows that progesterone blocks TNF- and thrombin-mediated fetal membrane attenuation by inhibiting monocyte/macrophage colony stimulating factor (GM-CSF) generation and

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activation. Thus, progesterone can prevent the preterm premature rupture of membranes, and plays a protective role in fetal membranes via anti-inflammatory effects.

Progesterone increased with increased gestational weeks. Therefore, compared to PPROM, the anti-inflammatory protective effect of progesterone is stronger in TPROM, indicating the stronger inhibition effect of progesterone on IL-1 and TNF production. Pathogen infection only enhances the activation of the pattern recognition receptors, such as the NLRP3, and increases the expression of cytokines, such as caspase-1 and IL-1, can destroy the cell structures composition, such as MMP-1, MMP-3, and induce cell apoptosis, eventually leading to rupture of membranes. In other words, the expression of inflammatory cytokines in patients with TPROM is higher than that in the PPROM patients. This may be one of the reasons for the differences in the expression of NLRP3 and caspase-1 in the fetal membranes and placental tissues in PPROM and TPROM patients. We plan to determine whether/how much progesterone affects the activation of NLRP3 and caspase3, and its role in PROM.

Results of this study show that the expression of NLRP3 and caspase-1 in fetal membrane and placental tissues may be associated with the occurrence and development of PROM, and we observed different expression between PPROM and TPROM patients. Our data provide a new potential target for the prevention and treatment of PROM. Further research is warranted on the expression of NLRP3 and caspase-1 in fetal membrane and placental tissues of reproductive tract-infected patients with or without PROM. To better predict and prevent PROM, it is necessary to find the boundary values of premature rupture of membranes. Because PROM is multi-factorial, involving both genetic and environmental factors, more factors should be considered in the occurrence of PROM, including genetics, ethnicity, and obesity, which are associated with inflammasomes. In the future, we will do in-depth research on the occurrence and development of PROM.

Conclusions

Our data show that NLRP3 and caspase-1 were significantly up-regulated in fetal membrane and placental tissues in PROM women, and this may be associated with the development of PROM.

Conflict of interest

None.

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