Original Article

Placental pathological manifestations and expression levels of apoptosis-related genes and HSP-70 in fetal distress and premature rupture of membranes

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Abstract: Objective: The present study was designed to investigate the pathological manifestations, cell apoptosis and the expression of heat shock protein (HSP)-70 in postpartum placenta of patients with fetal distress or premature rupture of membranes (PROM). Methods: The pathological changes, apoptosis and HSP-70 expression of placenta in 35 cases of fetal distress, 40 cases of premature rupture of membranes and 30 cases of normal delivery were retrospectively analyzed. Results: Compared with the normal placenta group, the placental pathological manifestations of the fetal distress group and the PROM group showed increased apoptosis of villous trophoblasts and decidual cells (P<0.001). Reverse transcription PCR (RT-PCR) revealed that the mRNA levels of apoptotic genes CDKN1A and BAX were significantly elevated (P<0.001). The expression level of HSP-70 in cells was also found to be markedly increased as measured by Western blot (P<0.01). Conclusion: Increased placental cell apoptosis may be involved in the pathological changes of fetal distress and PROM, in which the expression of apoptosis-related genes CDKN1A, BAX, and HSP-70 is also increased significantly.

Keywords: Fetal distress, premature rupture of membranes, pathological manifestations, apoptosis, heat shock protein-70

Introduction

Fetal distress and premature rupture of membranes (PROM) are common complications in the second and third trimester of pregnancy. According to the statistics, the two account for 1.5-5.6% among all the complications in the middle and terminal pregnancy, resulting in a neonatal mortality of 0.5-0.9%. Improper treatment will seriously affect the outcome of pregnancy and the safety of mother and baby. Although fetal distress and PROM are two independent diseases, recent studies have found that increased apoptosis of placental tissues caused by different degrees of immune rejection between the placenta and the mother may be an important mechanism for the occurrence of these diseases [1]. In addition, genetic defects, anatomical abnormalities of the reproductive system, endocrine disorders, infections, thrombus and abnormal in vivo and in vitro environment are also implicated in the occurrence of the diseases [2]. Placenta is an important bridge between the infant and the mother. Pathological observation of placental tissues after delivery has revealed that the occurrence of a variety of gestational diseases is related to the abnormal number and function of villous trophoblast cells and decidual cells [3], as well as the increase of cell apoptosis. Apoptosis is one of the ways of programmed cell death. Changes in the external environment activate apoptosis-related coordination and receptor binding, which in turn initiates the transcription and translation of apoptosis-related genes, and participates in the expression of various apoptotic proteins and active factors, such as CDKN1A, BAX, and heat shock protein-70 (HSP)-70 [4-6].

Increased placental cell apoptosis not only exists in the placental tissues of pregnant wo-
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...men with abnormal pregnancy, but also can be observed in the physiological processes of trophoblast cell apoptosis, blastocyst implantation and growth, degeneration and reconstruction of decidual tissues, as well as structural remodeling of placenta. Furthermore, with the continuous increase of gestational weeks, there are different pathological signs, and placental cell proliferation and apoptosis are often in a dynamic equilibrium. Once the apoptotic activity is higher than the proliferation, it may trigger a variety of pregnancy-related diseases [7]. What’s more, the expression activity of HSP-70 is enhanced in the hypoxic environment of cells, thus inducing cell damage repair, apoptosis and other adaptation to environmental changes. HSP-70 is highly conserved and mainly plays the role of “molecular chaperone”, which is bound up with cell proliferation and apoptosis. It has been found in in vitro culture of various tumor cells that HSP-70 can significantly facilitate cell apoptosis and inhibit cell proliferation under hypoxia environment. Based on this, the main purpose of this study was to explore the pathological manifestations, apoptosis and HSP-70 expression of placenta after delivery in patients with fetal distress or PROM.

Research participants and methods

Data of research participants

In this study, 35 cases of fetal distress, 40 cases of PROM, and 30 cases of normal pregnancy admitted to our hospital from August 2018 to August 2019 were enrolled for a retrospective analysis. Inclusion criteria: 1. Patients who met the diagnostic criteria for fetal distress and PROM based on the 9th edition of Obstetrics and Gynecology [8], those with normal pregnancy and without assisted reproduction; 2. All were pregnant with single live births, no twins or multiple pregnancies; 3. All were delivered successfully and the mother and baby were discharged safely; 4. The placenta was delivered intact after the fetus was delivered, and placental tissue sections could be prepared for subsequent research; 5. Informed consent was obtained and clinical data were complete. Exclusion criteria: 1. Patients with pregnancy complications, such as preeclampsia, gestational diabetes, placenta previa, or amniotic fluid abnormalities; 2. Congenital abnormalities in fetus; 3. Patients with severe liver and kidney dysfunction.

Research methods

After admission, patients with fetal distress or PROM actively cooperated to complete relevant examinations, closely monitored fetal intrauterine growth and labor progress, and tried to extend the gestational week as far as possible. Whereas, the pregnancy was terminated immediately in case the delivery conditions were appropriate or the risk of maternal and infant complications was high. According to the delivery conditions, natural delivery or cesarean section was properly selected to ensure the complete delivery of the placenta. The pathological manifestations of the placenta were observed under a light microscope, the apoptosis rate of placent al cells was detected by TUNEL method, the mRNA expression levels of apoptosis gene CDKN1A and BAX were measured by reverse transcription PCR (RT-PCR), and the expression of HSP-70 was determined by Western blot. As soon as the placenta was successfully delivered, it was fixed in 10% neutral formalin solution to prepare paraffin-embedded serial sections with a thickness of 3 μm and then placed in an oven at 70°C for 3 hours.

Detection of apoptosis rate by TUNEL method

Main steps: The placenta was pretreated with paraffin sections, followed by dewaxing, hydration and PBS washing. Then, the protease K solution (20 μg/mL, Sangon Biotechnology Co., Ltd., Shanghai, China) was added dropwise, and then 100 μL of it was incubated for 8 minutes at room temperature. On this basis, anti-fluorescein and TUNEL marker solution were added dropwise, and then washed 3 times with PBS, 5 minutes each. After equilibrating with buffer solution III, the color was developed at room temperature, and the nitroblue tetrazolium/bromochloroindolyl phosphate was added dropwise to observe the color.

RT-PCR detection of CDKN1A and BAX mRNA levels

The main steps were as follows: Firstly, the placental tissue cells were smashed by ultra-
sound and washed and dissolved by PBS solution. Then, RNA molecules were extracted from placental cells by one-step method according to TRIzol kit (Invitrogen Company, USA). After determining the concentration and purity of RNA, cDNA was synthesized by reverse transcription kit (Dalian TaKaRa, China). The target primer sequence was synthesized by Takara Company, Japan, with reference to Gene Bank (Table 1). A PCR amplification instrument (MJ research company, USA) was adopted to configure the reaction system according to the reaction requirements, including SYBR® Premix Ex Taq™ (2×): 12.5 µL + upstream and downstream primers 10 µM: 1 µL each, and reaction water was added to make up to a total volume of 25 µL. The reaction parameters were set at 92°C for 20 s (96°C for 2 s, 85°C for 20 s, and 80°C for 6 s) for a total of 30 cycles. The fluorescence was read at 75°C to construct a dissolution curve, and the relative expression of target primer mRNA was calculated by 2^ΔΔCt method.

Detection of HSP-70 expression by Western blot

Placenta tissue was rapidly homogenized by ultrasound and then added with RIPA lysate (Sigma, USA) to extract the total cell protein. BCA protein quantitative kit (Sigma, USA) was applied to detect concentration and purity. Then 30 µg sample protein was taken from each group and separated by 8% SDS-PAGE electrophoresis before the charged separation area was transferred to PVDF membrane, followed by the dropwise addition of rat anti-human HSP-70 and β-actin primary antibody (1:2000, sigma company of America) in sequence and left to stand overnight. After washing with PBS, rabbit anti-rat secondary antibody (1:500, sigma, USA) was added and incubated at room temperature for 4 h, washed with PBS, and ECL was used for color development. The results were scanned and saved, and a semi-quantitative analysis was performed by Lab Works 4.5 gel imaging software (Invitrogen, USA). The results were expressed as the gray value of HSP-70/β-actin band.

Statistical methods

SPSS 20.0 statistical software (SPSS Inc., Chicago, IL, USA) was used to represent the measurement data between the three groups as the mean ± standard deviation. ANOVA test was employed for comparison, and LSD-t method was applied for pairwise comparison. The counting data was expressed as the number/percentage (n/%), and the comparison between groups was conducted by X^2 test. P<0.05 was considered to be statistically significant.

Results

Comparison of baseline data

The baseline data of the three groups of pregnant women and fetuses were comparable (P>0.05) (Table 2).
Postpartum placenta of pregnant women with fetal distress or PROM

Table 3. Comparison of apoptosis rate of villous trophoblasts and decidual cells in placenta of each group (%)

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of cases</th>
<th>Villous trophoblast</th>
<th>Decidual cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal distress</td>
<td>35</td>
<td>36.2±6.9***</td>
<td>41.2±10.3***</td>
</tr>
<tr>
<td>Premature rupture of membranes</td>
<td>40</td>
<td>35.6±7.8***</td>
<td>40.5±9.6***</td>
</tr>
<tr>
<td>Normal pregnancy</td>
<td>30</td>
<td>5.6±1.3</td>
<td>5.9±1.4</td>
</tr>
<tr>
<td>F</td>
<td>65.639</td>
<td>89.524</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
</tr>
</tbody>
</table>

Note: ***compared with the normal placenta group, P<0.001.

Pathological manifestations of placenta in each group

Compared with the normal placenta group, the placental pathological manifestations of the fetal distress group and the PROM group showed increased apoptosis of villous trophoblasts and decidual cells (P<0.001) (Table 3; Figures 1, 2).

Typical legend analysis: Clinical diagnosis of Figure 2B: Preterm PROM, visible to the naked eye: one placenta, 17.5×14.5×6.3 cm in volume, smooth fetal surface, rough maternal surface, 1.5 cm distance from the rupture of fetal membrane to the edge of placenta, grayish red and grayish yellow, thickness of about 0.1 cm. A umbilical cord lies in the center of the placenta with a length of 32.4 cm, maximum diameter of 1.5 cm, grayish white, coagulation in the cut cavity, placenta with multiple sections, purple sponge like, soft. Diagnosis: Degeneration of placental villi with fibrinoid deposition and calcification, chronic inflammation of membrane, no obvious abnormality in umbilical cord.

Clinical diagnosis of Figure 2C: Fetal distress, visible to the naked eye: one placenta, 19.5×16.4×6.5 cm in volume, smooth fetal surface, rough maternal surface, 6.5 cm away from the edge of placenta, grayish red and grayish yellow, about 0.1 cm thick. A umbilical cord in the center of placenta, 10.3 cm long, 1.9 cm in maximum diameter, grayish white, coagulation in the section cavity, multiple sections of placenta, purplish red sponge like, soft. Diagnosis: Placental villus degeneration with fibrinoid deposition, chronic inflammation of fetal membrane with focal acute inflammatory cell infiltration, no obvious abnormality in umbilical cord.

Discussion

Fetal distress and PROM are self-protective responses of the placenta due to changes in the external environment during pregnancy, involving a variety of pathological mechanisms, such as increased apoptosis of placental cells, aggravation of hypoxia, disturbance of inflammatory reaction or oxidative stress, and abnormal repair of injury [3]. The placenta tissue can release a variety of active factors to participate in the process of apoptosis and hypoxia adaptation. As to apoptosis, it happens throughout the normal pregnancy, but it is mainly concentrated in the trophoblast and syncytiotrophoblast [4]. All these suggest that apoptosis of placental cells may be a common phenomenon, and abnormal apoptotic activity may be one of the important mechanisms of various pregnancy diseases [5, 6].

In this study, pathological observation was made on the placental tissues of fetal distress and PROM and the apoptotic activity was detected. It was found that the apoptotic activities of villous trophoblast cells and decidual cells in the two gestational diseases were abnormally enhanced and the apoptotic distribution was larger compared with the normal gestational group. At 4-5 weeks of normal gestation, the apoptotic activities of placental cytotrophoblast cells and syncytiotrophoblast cells increase abnormally, which is a major cause of early abortion and the main manifes-
Postpartum placenta of pregnant women with fetal distress or PROM

Figure 1. TUNEL method to detect the apoptotic rate of cells in placental tissue of each group (100×). A. Normal placenta. B. Fetal distress. C. Premature rupture of membranes. The staining of apoptotic cells (black arrows) ranges from dark brown to light brown. Apoptosis mainly occurs in trophoblastic epithelial cells (apoptotic nucleus concentration, obvious edge aggregation) and crypt epithelial cells (no obvious shrinkage in nucleus and cytoplasm). FCV: trophoblastic epithelial cells; MCC: crypt epithelial cells.

Figure 2. HE staining of placental tissue in each group (100×). A. Normal gestational placenta. B. Fibrinoid deposition and calcification, as well as the changes in bleeding can be seen in the placental tissues of patients with premature rupture of placenta. → represents fibrinoid deposition. C. Fibrinoid deposits and inflammatory cell infiltration can be seen in the placental tissues of fetal distress patients. → represents inflammatory cell infiltration.

Table 4. Comparison of mRNA expression of apoptotic genes in each group

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of cases</th>
<th>CDKN1A</th>
<th>BAX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal distress</td>
<td>35</td>
<td>0.3569±0.0857###</td>
<td>0.4125±0.1052###</td>
</tr>
<tr>
<td>Premature rupture of membranes</td>
<td>40</td>
<td>0.3659±0.0968###</td>
<td>0.4215±0.1023###</td>
</tr>
<tr>
<td>Normal pregnancy</td>
<td>30</td>
<td>0.1236±0.0532</td>
<td>0.1152±0.0424</td>
</tr>
<tr>
<td>F</td>
<td></td>
<td>102.326</td>
<td>156.236</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Note: ### compared with the normal placenta group, P<0.001.

Table 5. Comparison of HSP-70 expression in each group

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of cases</th>
<th>HSP-70/β-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal distress</td>
<td>35</td>
<td>0.51±0.11##</td>
</tr>
<tr>
<td>Premature rupture of membranes</td>
<td>40</td>
<td>0.89±0.22##,**</td>
</tr>
<tr>
<td>Normal pregnancy</td>
<td>30</td>
<td>0.29±0.01</td>
</tr>
<tr>
<td>F</td>
<td></td>
<td>53.263</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>0.000</td>
</tr>
</tbody>
</table>

Note: ## compared with the normal placenta group, P<0.01; ** compared with the fetal distress group, P<0.01.
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The evolution of apoptotic activity of placental cells throughout pregnancy is also a manifestation of biological functions of placenta formation, development, and delivery [10, 11]. Furthermore, we found that the mRNA levels of apoptotic genes CDKN1A and BAX in the placental tissue of the fetal distress group and the PROM group were significantly increased. CDKN1A protein is one of the key downstream transcripts of TP53 gene [12, 13]. Studies have confirmed that TP53-CDKN1A pathway regulated by human rpl3 gene can affect cell proliferation and transcriptional cycle and promote cell apoptosis [14]. While BAX has a pro-apoptotic effect and can directly initiate the mitochondrial related apoptotic pathway under the activation of TP53 [15]. BCL2 and BAX are essential molecular markers that reflect the apoptotic activity and direction of the cells. As the main anti-apoptotic molecule, BCL2 often indicates the apoptotic process of cells with the percentage of BCL2/BAX. If the percentage exceeds 50%, it indicates that the apoptotic activity of cells is weakened [16, 17].

Moreover, it was observed that the expression of HSP-70 in placenta was also remarkably elevated in the fetal distress group and the PROM group. HSP70 belongs to a multi-gene, multi-structure and multi-function family, consisting of 21 proteins [18]. It can promote or inhibit apoptosis under different conditions, and the apoptosis mediated by it can play a role through mitochondrial pathway, JNK signaling pathway and by affecting the protein conformation necessary for cell proliferation [19, 20]. HSP-70 is abundantly expressed in human reproductive system, and it is more expressed in endometrium, decidua and fallopian tubes [21]. It is significantly higher in the secretory phase of endometrial gland cells than in the proliferative phase, and is mainly localized in the cytoplasm. The placenta can also express plentiful HSP-70 in hypoxia or stress environment, particularly in the syncytiotrophoblast layer. The placenta in the third trimester of pregnancy is subject to more in vivo and in vitro interference factors, therefore, the secretion activity of HSP-70 also changes significantly [22]. The quantity and quality of HSP-70 expression in placental tissues of spontaneous abortion, fetal distress and PROM were significantly different from those of normal pregnancy [23]. Its overexpression not only causes local ischemia of decidual tissue and restriction of embryonic growth, but also activates immune cells by affecting hormone regulation, resulting in abnormal immune rejection between decidual tissue and maternal-fetal interface, which ultimately leads to pregnancy failure or abnormal fetal development [24, 25]. HSP-70 can also continuously induce placental inflammatory response [26]. Increased expression of HSP-70 in decidual tissues indicates that the body is suffering from hypoxia or a severe stress response, which is more susceptible to endocrine hormone disorders and uterine sensitivity enhancement, leading to severe pregnancy complications such as abortion, fetal distress and PROM [27].

The innovation of this study is to explore the relationship between apoptosis and apoptosis-related expression of genes or active proteins.
and the occurrence of fetal distress and PROM from the placental microcosmic point of view, which provides vital references for the pathogenesis of the diseases. However, there is still room for improvement. There may be more mechanisms involved in fetal distress and PROM, whether increased apoptosis of placenta cells, abnormal expression of apoptotic genes CDKN1A, BAX and HSP-70 play a major role, and their correlations with other mechanisms remain to be further explored.

Taken together, increased apoptosis of placental cells may be involved in the pathological changes of fetal distress and PROM, in which the expressions of apoptosis-related genes CDKN1A, BAX, and HSP-70 also increase significantly.

Disclosure of conflict of interest

None.

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