Original article

Effect of IL-18 on intrauterine infection of HBV in mice on cell molecular level

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Abstract

Objective: The objective of this study is to investigate the effect of IL-18 on intrauterine infection of HBV (Hepatitis B Virus) in mice based on cellular and molecular level, and to analyze its mechanism, as well as the relationship between IL-18 and intrauterine infection of HBV.

Methods: Pregnant rats are taken as the study subjects and divided into two groups according to infection and non-infection, namely the study group and the control group. Firstly, the peripheral blood of rats and the blood of newborn mice are collected for the determination of hepatitis B in two-and-a-half pairs. Then, the levels of interleukin-18 (IL-18), interferon-γ (IFN-γ) and interleukin-4 (IL-4) in peripheral serum are detected by ELISA (Enzyme Linked Immunosorbent Assay). Finally, the two groups of horizontal values are compared and analyzed. The effect of IL-18 on intrauterine infection of HBV in mice is investigated based on the level of cell and molecular.

Results: The levels of IL-18, IFN-γ, IL-4 and IFN-γ/IL-4 in the two groups are compared and analyzed. The levels of IL-18, IFN-γ and IFN-γ/IL-4 in the study group are significantly lower than those in the control group, with statistical significance. However, the level of IL-4 in the study group is higher than that in the control group, with statistical significance.

Conclusion: It is found that the decrease of HL-type specific response and the enhancement of Th2-type specific response in pregnant mice are closely related to HBV intrauterine infection. Moreover, the decrease of IL-18 secretion in peripheral blood may cause intrauterine infection of HBV. This study can make people better realize the mechanism of HBV intrauterine infection, and effectively help clinical prevention and treatment of intrauterine infection.

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1. Introduction

The World Health Organization lists hepatitis B virus (HBV) infection as one of the ten leading causes of death in the world, which is a serious public health problem in the world (Makhlouf et al., 2019). About 350 million people worldwide are infected with HBV. There are 112 million people in China who are chronically infected with HBV, accounting for 33% of the world’s infected people, which is a high incidence area of hepatitis B, and this number is on the rise (Dakic et al., 2019). Mother-to-child transmission of HBV is one of the important ways of transmission of HBV in China. HBV infection caused by mother-to-child transmission accounts for about 1/3 of infant infection in China (Avadhanula et al., 2015). Mother-to-child transmission mainly includes intrauterine transmission, intrapartum transmission and postpartum transmission (Zeng et al., 2015). Recent studies have shown that HBV intrauterine infection is as high as 9.1%–36.7% (Liu et al., 2018). Intrauterine infection of HBV is the main route of mother-to-child transmission, which is the main reason for the failure of postnatal hepatitis B vaccine (HBVac) vaccination. Therefore, blocking mother-to-child transmission is the key link to control the epidemic of hepatitis B (Wahid, 2020). However, the mechanism of HBV intrauterine infection is still not very precise.

In recent years, with the development of molecular biology technology and Immunology theory, it has been recognized that HBV infection of host cells is a complex internalization process involving multiple cytokines. Moreover, host acquired immune balance regulated mainly by helper T lymphocyte-1 (Th1) and helper
T lymphocyte-2 (Th2) cells and their cytokines plays an important role in the occurrence and prognosis of viral infection (Meng et al., 2015). Interferon-γ (IFN-γ) is a characteristic factor of Th1 cells. In addition to enhancing the anti-virus ability of the body, IFN-γ also has an important role in immune regulation (Zhou et al., 2015). Interleukin-4 (IL-4) is a characteristic factor of Th2 cells, which dominates the body's humoral immunity. The researchers believe that IFN-γ/IL-4 is a method of Th1/Th2 response mode in HBV infection (Yi et al., 2016). If the level of IFN-γ in umbilical cord blood of newborns with HBV intrauterine infection is low and IL-4 level is elevated, the Th1 cell immune response of the body will be down-regulated while the Th2 cell immune response will be relatively enhanced, the antiviral ability of pregnant women will be weakened, and the replication of HBV will be active, which makes the virus easy to pass through the placental barrier and lead to intrauterine infection of the fetus. Among many cytokines, the role of HBV is mainly focused on IFN-γ, IL-4 and IL-18.

To further explore the effect of IL-18 on intrauterine infection of HBV in mice, pregnant mice are taken as the research objects to analyse the mechanism of intrauterine infection of HBV based on the level of cellular molecular analysis, and the relationship between IL-18 and intrauterine infection of HBV. Through this study, people can understand the mechanism of HBV intrauterine infection more clearly, which is of great significance in clinical prevention and treatment of intrauterine infection.

2. Materials and methods

2.1. Research object

101 pregnant female Balb/c rats weighing 23.5 ± 1.5 g are selected as experimental subjects in 8–10 weeks. All animals were fed with national standard rodent in separate cages, 4 animals in each cage, free diet and drinking water, no significant difference in body weight between groups. They had natural light, free diet, room temperature controlled at 20–26 °C, humidity controlled at 40–50%, and adaptive feeding for 2 weeks. The experimental procedure is approved by the Ethics Committee, and the experimental sequence conforms to the national experimental animal standards.

Grouping: The 101 mice are divided into two groups. Among them, 21 cases of HBV intrauterine infection are set as the study group, and 80 cases of non-HBV intrauterine infection are set as the control group.

3. Laboratory reagents and equipment

3.1. Collection and preservation of specimens

Peripheral blood of 3 ml rats is collected and centrifuged in an automatic centrifuge of 3000 rpm for 30 min. Then, it is frozen in an EP tube at −20 °C refrigerator, waiting for cytokines IL-18, IFN-γ and IL-4 to be detected. Immediately after birth, the blood of the newborn mice is collected in two tubes, each of which is 3 ml, and centrifuged in an automatic centrifuge of 2000 rpm for 20 min. Serum is separated. One tube is used to detect two-and-a-half pairs of hepatitis B, and the other tube is used to detect HBV-DNA. In order to avoid the contamination of the blood collection site by maternal substances, it is necessary to scrub the blood collection site with normal saline before collecting blood, and then wipe it with alcohol.

3.2. Two-and-a-half determination of hepatitis B

ELISA is adopted. HBs Ag is taken as an example. Other serum markers of hepatitis B are similar.

From the sealed bag of the kit which has been balanced to room temperature, the required reaction strips and reagents are removed, and the concentrated detergent is diluted with double distilled water at 1:25 and then loaded into the washing machine. 50 μl/hole is added into the pore of the reaction plate. At the same time, two holes of negative control, positive control and blank control are set up (negative and positive control holes are added to corresponding control serum 50 μl, blank control holes are added to 100 μl detergent), then enzyme marker 50 μl/hole (blank holes are not added) is added to mix. After laminating, the laminate is kept at 37 °C for 30 min. Washing board: After discarding the liquid in the board, the washing board machine is used to wash the reaction board and remove water droplets (pat dry on the thick stack of absorbent paper). It is washed five times. Color rendering and termination: Substrates A and B are added with 50 μl/hole each. After 10 min of color rendering at 37 °C, 50 μl/hole of terminating solution is added. Determination and result judgment: OD (Optical Density) value of each pore is measured by the enzyme labeling instrument at 450 nm single wavelength. The OD value of the negative control hole is used as the reference to calculate the critical value, and then the OD value of each specimen is measured according to the experiment to judge whether the result is negative or positive.

3.3. Detection of HBV-DNA in serum of neonatal mice by fluorescence quantitative PCR

Specimen processing: 40 μl of serum samples are taken out and mixed with the same amount of DNA extract. After 10 min of boiling water bath, 10000 rpm centrifugation is needed for 5 min and supernatant 2 μl is taken for PCR reaction. Standard dilution and treatment: The centrifugal number of positive quantitative quality control standard (1 × 10⁶ copi/ml) 6000 rpm is 10⁴. Four new 0.5 ml sterile centrifugal tubes need to be taken and each 45 μl negative control substance is added respectively, which are marked 10⁻²⁻¹⁰⁴ in turn. Pipes 5 μl 10⁻⁶ to 10⁻¹ need to be sucked. When the sampler is used for repeated mixing, a new suction head is needed to take 5 μl to 10⁻⁴ tubes. The diluted positive standard gradient and negative control 40 μl are absorbed respectively. The same amount of DNA extract is added and evenly beaten. The following steps are the same as above. PCR amplification: In several HBV-PCR reaction tubes, the treated samples or negative and positive quality control standard 2 μl, 6000 rpm centrifugation for 1 min are added directly. Each reaction tube is placed in the reaction tank of the quantitative PCR instrument. Negative control substance, gradient of positive quantitative quality control standard substance and unknown sample are set in corresponding order. Sample names are set to label the fluorescent group types and cycling conditions: ABI PRISM 7000, ABI Gene Amp5700 cycling conditions: 93 °C → 2 min pre-denaturation, then 93 °C, 45 s → 55 °C, 60 s. 10 cycles are made first, then 30 cycles are made at 93 °C, 30 s → 55 °C, 45 s. Roche Light Cycler cycle conditions: 93 °C, 5 s to 2 min of pre-denaturation, and then according to 93°, 5 s → 57/45 s, 40 cycles is made. The setting of results analysis conditions: ABI PRISM 5700, ABI Gene Amp7000: According to the image after analysis, the start value (2–4), stop value (7–9) and threshold value of baseline is adjusted. The standard curve under Std Curve window is the best. Finally, under the Reporter window, the unknown sample value (Qty) calculated by automatic analysis of the instrument is recorded. Conditions for Roche Light cycle: After the reaction, the data file is automatically saved and detected, and the fluorescence value is adjusted to F1/F2. Quantification is clicked to read the results. The noise tolerance is adjusted above the negative control. Under Step3: Analysis, the correlation R value is required to be less than −0.57, close to −1.0. Negative control CT value guarantees no value. Finally, the unknown sample value (M) automatically analyzed by the instrument is recorded. Calculations
of the results: ABI PRISM 5700, ABI GeneAmp7000 instrument: If the CT value is less than 30, the HBV DNA content (gene copy number/ml) of the experimental sample is equal to Qty. If the CT value is 30, the HBV DNA content (gene copy number/ml) is less than 1 × 10^3. Roche Light cycle Instrument: If the CT value is less than 40, the HBV DNA content (gene copy number/ml) of the experimental sample is M. If the CT value is 40, the HBV DNA content (gene copy number/ml) is less than 1 × 10^3. The quantitative determination range of HBV-DNA is 10^{3.0−9.5} copy/ml.

3.4. Determination of cytokines

Using ELISA quantitative method, the detection of IFN-γ is taken as an example. The detection of IL-18 and IL-4 is similar. The required reaction strips and reagents is removed from the sealed bag of the kit which has been balanced to room temperature, and the concentrated detergent is diluted with double deionized water at 1:10 and then it is put into the washing machine. The pore of the reaction plate is mixed with 5 μL standard and 5 μL of serum for 10 s. Each hole is added to 200 μL biotin IFN-γ, and then it is incubated for 30 s. After laminating, it is incubated at 37 °C for 30 min. Washing board: After discarding the liquid in the board, the washing board machine is used to wash the reaction board and remove water droplets (pat dry on the thick stack of absorbent paper). It is washed five times. Each hole is added with 200 μL HRP and blended gently for 10 s. After laminating, the laminate is placed at 37 °C for 30 min. Washing board: After discarding the liquid in the board, the washing machine is used to wash the reaction board and remove water droplets (pat dry on the thick stack of absorbent paper). Each hole is added with 100 μL TMB color solution, gently mixing for 10 s. After laminating, it is placed in a dark place and incubated at room temperature for 20 min. Each hole is added with Stop Solution and blended gently for 30 s. Reading OD value: After adding termination solution, the OD value needs to be read by enzyme labeling instrument at 450 nm single wavelength within 15 min. If the OD value of the specimen is higher than the upper limit of the standard curve, it should be diluted properly and retest. When calculating the concentration, the dilution factor should be multiplied. The standard curve: OD value of standard product is taken as variable and concentration as strain. The SPSS 22.0 for windows statistical software package is used to carry out statistical analysis by linear regression, square regression, cubic regression and logarithmic regression. The curve conforms to regression equation: concentration = a*OD + b*OD + c.

3.5. Statistical methods

The results are expressed as X ±S. SPSS 22.0 for windows statistical software package is used for statistical analysis in data processing and analysis. The statistical methods used included curve regression, t-test, chi-square test and Pearson correlation analysis. The differences of IL-18, IFN-γ, IL-4 secretion levels and IFN-gamma/IL-4 ratio between pregnant women with and without HBV intrauterine infection and the correlation between IL-18 and IFN-γ, IL-4, IFN-γ/IL-4 and HBV intrauterine infection are discussed. Statistical significance level is set to P < 0.05.

3.6. Observation index

HBV intrauterine infection criteria: HBs Ag and/or HBV-DNA are detected in the serum of newborn mice immediately after birth (HBV DNA > 1.00 × 10^3 copies/ml is positive). Levels of cytokines IL-18, IFN-γ and IL-4: The OD values and regression curves of cytokines IL-18, IFN-γ and IL-4 in the two groups are measured by experiment, and the levels of cytokines are calculated.

4. Results

4.1. Comparison of general data between study group and control group

The comparison of general data between the study group and the control group is shown in Tables 3 and 4. There is no significant difference in age, parity, gestational week, gender of newborn mice and mode of delivery between the two groups (P > 0.05). There is the comparability between the two groups, as shown in Tables 1 and 2.

4.2. Intrauterine infection rate of HBV

In this study, 101 pregnant rats with HBs Ag and HBeAg are given birth to 101 newborn mice. 8 cases are the serum HBV-DNA positive and its rate is 17.82% (18/101). 8 cases are HBs Ag

### Table 1

| Name | Place of origin |
|------|----------------|
| ELISA Two-and-a-half Detection Kit for Hepatitis B | Zhongshan Bioengineering Co., Ltd. |
| Fluorescence Quantitative Detection Kit for HBV (HBV) Nucleic Acid Amplification (PCR) | Sun Yat-sen University Daan Gene Co., Ltd. |
| ELISA IL-18 Quantitative Detection Kit | RapidBio Lab, Calabasas California USA |
| ELISA IL-4 Quantitative Detection Kit | Rapid Bio Lab, Calabasas California USA |
| ELISA-IFN-γ Quantitative Detection Kit | Rapid Bio Lab, Calabasas California USA |

### Table 2

| Name | Place of origin |
|------|----------------|
| LabSystems MK3 Enzyme Marker | Thermo LabSystems, Finland |
| DNX-9620 Computer Plate Washer | Beijing Puling New Technology Co., Ltd. |
| Adjustable multi-channel pipette (5–200 μL) | Thermo LabSystems, Finland |
| – 200 °C Electronic Temperature Controlled Cryogenic Refrigerator | German Siemens |
| LIGHTCYCLER Fluorescence Quantitative PCR System | Switzerland Roche |
| Automatic centrifuge | Germany Sigma |
| WS2-261-79 Electronic Constant Temperature Water Bath | Shanghai Medical Thermostatic Equipment Factory |
| ZDQ-III Vortex Oscillator | Changchun Boyan Scientific Instruments Co., Ltd. |
positive, and the positive rate is 7.92% (8/101). 21 cases are HBsAg and/or HBV-DNA positive, and the intrauterine infection rate is 20.79% (21/101).

4.3. Comparison of serum IL-18 levels in pregnant rats

The detection results of the peripheral serum IL-18 level of the two groups of pregnant rats are shown in Fig. 1. The peripheral serum IL-18 level of the two groups of pregnant rats is obtained (Fig. 1B) by the calculation equation from the measured standard curve of IL-18 concentration (Fig. 1A). The level of IL-18 in peripheral blood of pregnant rats in the study group is 802.71 ± 134.65 pg/ml, which is significantly lower than that of the control group (905.93 ± 170.35 pg/ml). There is a significant difference between the two groups, t = 2.57, P < 0.05, as shown in Table 5.

4.4. Comparison of serum IFN-γ and IL-4 levels and IFN-γ/IL-4 ratio in pregnant rats

The detection results of IFN-γ and IL-4 levels in the peripheral serum of the two groups of pregnant rats are shown in Fig. 2. The regression curve between the measured IFN-γ and IL-4 concentrations and the experimental OD value (Fig. 2A and B) is used to obtain the comparison of the levels of IL-18 and the ratio of IFN-γ/IL-4 in the peripheral serum of the two groups of pregnant rats (Fig. 2C). The level of IFN-γ in peripheral blood of pregnant rats in the study group is 468.47 ± 86.93 pg/ml, which is significantly lower than that of the control group (547.42 ± 107.32 pg/ml). There is significant difference between the two groups, t = 3.11, P < 0.01.

The level of IL-4 in peripheral blood of pregnant rats in the study group is 133.96 ± 51.18 pg/ml, which is significantly higher than that of the control group (108.42 ± 28.83 pg/ml). There is significant difference between the two groups, t = 2.198, P < 0.05.

The IFN-γ/IL-4 ratio in peripheral blood of pregnant rats in the study group is 4.07 ± 1.88, which is significantly lower than that of the control group (5.48 ± 1.99). There is significant difference between the two groups, t = 2.915, P < 0.01, as shown in Table 6.

4.5. Analysis of correlation between serum IL-18 and IFN-γ and IL-4 levels in pregnant rats

The comparison results of the levels of IL-18, IFN-γ and IL-4 in the peripheral serum of the two groups of pregnant rats are shown in Fig. 3. Pearson correlation analysis is used to analyze the levels of IL-18, IFN-γ and IL-4 in peripheral blood of pregnant rats in the study group and control group. It is found that there is a significant positive correlation between IL-18 and IFN-γ in the serum of pregnant rats in each group. The correlation coefficient r is 0.747 and 0.911 respectively (P < 0.01, P < 0.01). IL-18 and IL-4 secretion levels are negatively correlated. The correlation coefficients r are −0.640 and −0.298 (P < 0.01, P < 0.01), as shown in Tables 7 and 8.

4.6. Analysis of correlation between serum IL-18 level and IFN-γ/IL-4 ratio in pregnant rats

Pearson correlation analysis is used to analyze the ratio of IL-18 to IFN-γ/IL-4 in peripheral blood of pregnant rats in study group and control group. The correlation coefficients of IL-18 and

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Table 3

| Grouping | Cases | Age     | Gestational week | Gravida | Delivery times |
|----------|-------|---------|------------------|---------|----------------|
| study group | 21    | 26.71 ± 4.52 | 39.76 ± 1.33 | 2.33 ± 1.19 | 0.43 ± 0.59 |
| control group | 80    | 25.61 ± 4.11 | 39.35 ± 1.42 | 2.03 ± 1.27 | 0.41 ± 0.61 |
| t         |       | 1.071    | 1.175           | 1.000   | 0.108          |
| P         |       | 0.287    | 0.243           | 0.320   | 0.914          |

Table 4

| Clinical data | Cases | Neonatal male mice |
|---------------|-------|---------------------|
| study group   | 21    | 12(56.25%)          |
| control group | 80    | 13(41.25%)          |
| X²            |       | 0.005               |
| P             |       | 0.941               |

Table 5

| Grouping | Cases | IL-18 (pg/ml) |
|----------|-------|---------------|
| study group | 21    | 802.71 ± 134.65 |
| control group | 80    | 905.93 ± 170.35 |
| t         |       | 2.571         |
| P         |       | 0.012         |
IFN-\(\gamma\) and IL-4 levels in peripheral blood of two groups of pregnant rats (A: regression curve of IFN-\(\gamma\) concentration and experimental OD value; B: regression curve of IL-4 concentration and experimental OD value; C: comparison of IFN-\(\gamma\), IL-4 levels and IFN-\(\gamma\)/IL-4 ratio).

**Table 6**
Comparison of peripheral serum IFN-\(\gamma\) and IL-4 levels and IFN-\(\gamma\)/IL-4 ratios between the two groups.

| Grouping          | Cases | IFN-\(\gamma\) (pg/ml) | IL-4 (pg/ml) | IFN-\(\gamma\)/IL-4 |
|-------------------|-------|-------------------------|--------------|---------------------|
| study group       | 21    | 468.47 ± 86.93          | 133.96 ± 51.18 | 4.07 ± 1.88         |
| control group     | 80    | 547.42 ± 107.32         | 108.42 ± 28.83 | 5.48 ± 1.99         |
| \(t\)             |       | 3.110                   | 2.198        | 2.915               |
| \(P\)             |       | 0.002                   | 0.038        | 0.004               |

**Table 7**
Pearson correlation analysis of IL-18 and IFN-\(\gamma\) and IL-4 levels in the study group.

|            | IFN-\(\gamma\) | IL-4 |
|------------|----------------|------|
| IL-18      | 21             | 21   |
| Correlation coefficient \(r\) | 0.747 | -0.640 |
| \(P\) value | <0.001 | 0.002 |

**Table 8**
Pearson correlation analysis of IL-18 and IFN-\(\gamma\) and IL-4 levels in control group.

|            | IFN-\(\gamma\) | IL-4 |
|------------|----------------|------|
| IL-18      | 80             | 80   |
| Correlation coefficient \(r\) | 0.911 | -0.298 |
| \(P\) value | <0.001 | 0.007 |

**Table 9**
Analysis of Pearson correlation between IL-18 and IFN-\(\gamma\)/IL-4 in study group.

|        | Cases | IFN-\(\gamma\)/IL-4 |
|--------|-------|---------------------|
| IL-18  | 21    | Correlation coefficient \(r\) 0.838 |
|        |       | \(P\) value <0.001 |

IFN-\(\gamma\)/IL-4 are 0.838, 0.634, \(P < 0.01\) and \(P < 0.01\), respectively, as shown in Tables 9 and 10.
5. Discussion

In recent years, the incidence of HBV is getting higher and higher (Al Awaidy and Ezzikouri, 2020). The main reason is vertical transmission between mother and infant. According to research reports, nearly half of the patients are related to vertical transmission of mother-to-child (Eswaraiah et al., 2020). The intrapartum and postpartum transmission can be controlled by the combination of hepatitis B vaccine and hepatitis B high-titer immunoglobulin. Although it can be effectively alleviated, it cannot completely block the route of transmission. Previous studies have found that the incidence of intrauterine infection is low, but in recent years, the incidence has been increasing, and has become the main way of vertical transmission of mother-to-child. Therefore, it is necessary to analyze its pathogenesis and the relationship between IL-18 and HBV intrauterine infection based on cytoregulatory research.

Pregnant mice were used to explore the effect of IL-18 on HBV intrauterine infection. It was found that the levels of IL-18, IFN-γ and the ratio of IFN-γ/IL-4 in HBV intrauterine infected pregnant mice were significantly lower than those in uninfected pregnant mice, and there was a significantly positive correlation between the levels of IL-18 and IFN-γ, and a significantly negative correlation between the two groups. The results showed that IFN-γ level of Th1 cells decreased with the decrease of IL-18 level. The level of IL-4 secreted by Th2 cells was relatively high, the immune response of Th1 cells was down-regulated while that of Th2 cells was relatively enhanced, and the immune response of Th1/Th2 cells was unbalanced. The study showed that IL-18 can significantly increase the expression of IFN-γ in PBMC of normal people, while the expression level of IL-18 in asymptomatic carriers and chronic hepatitis B patients in remission period is low (Dakic et al., 2019; Tian et al., 2019). It is speculated that the persistent infection state of asymptomatic carriers is related to the fact that the low level of IL-18 cannot effectively promote the Th1 response to eliminate the virus. Although the decrease of IL-18 level does not have the specificity of HBV intrauterine infection, the change of IL-18 level can indirectly reflect the degree of host immune imbalance. The low level of IL-18 can reduce IFN-γ production and decrease IL-4 secretion, and decrease FasL-mediated cytotoxic effect, thus affecting the immune function of pregnant rats and making HBV break through the placental barrier and cause intrauterine infection under the condition of imbalance of Th1/Th2 cell immunity in pregnant rats. Hence, the level of IL-18 in peripheral blood of HBV pregnant mice decreased, the possibility of HBV intrauterine infection increased, and the low level of IL-18 participated in the mechanism of HBV intrauterine infection.

In conclusion, the decrease of Th1 specific response and the increase of Th2 specific response in pregnant rats are closely related to HBV intrauterine infection, and the decrease of IL-18 secretion in peripheral blood will cause HBV intrauterine infection. Through this study, people can more clearly understand the mechanism of HBV intrauterine infection, which has great significance in clinical prevention and treatment of intrauterine infection.

6. Conclusion

The mechanism of intrauterine infection and the relationship between IL-18 and intrauterine infection of HBV are analyzed. It is found that the decrease of Th1 specific response and the increase of Th2 specific response in pregnant rats are significantly related to HBV intrauterine infection, and the decrease of IL-18 secretion in peripheral blood would cause HBV intrauterine infection. This study provides a reference for the mechanism of HBV intrauterine infection, which is helpful for clinical prevention and treatment of intrauterine infection. However, there are also some shortcomings in the research process. For instance, the small amount of data collected from the samples leads to a certain deviation of the results. Thus, in the later research process, the data capacity will be further increased, so that the results obtained are more valuable for reference.

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