Hepatitis B Virus Expression and Replication in Ovum and the Influencing Factors

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ABSTRACT

Background/Aim: The aim of this study was to investigate the factors that influence hepatitis B virus (HBV) expression and replication in the ovum. **Materials and Methods:** Immunohistochemistry and *in situ* hybridization techniques were used to assay the distributions of HBcAg, HBV DNA, and HBV mRNA in ovarian tissues and the ovum in 50 patients with chronic HBV infection. HBeAg and HBV DNA in the serum were also detected. Comparisons of categorical data were performed using McNemar test. **Results:** The positive rates of HBcAg, HBV DNA, and HBV mRNA in ovum and ovarian tissues of high replication group were significantly higher than low replication group (χ^2 = 15.04, P < 0.05; χ^2 = 12.96, P < 0.05; χ^2 = 19.36, P < 0.05; respectively). High positive rates of HBcAg, HBV DNA, and HBV mRNA in ovum and ovarian tissues were found in women with HBeAg-positive than HBeAg-negative (χ^2 = 113.14, P < 0.05; χ^2 = 11.13, P < 0.05; χ^2 = 17.39, P < 0.05; respectively). **Conclusion:** HBV can infect and replicate in the ovary and ovum. Maternal HBeAg status and HBV DNA levels are important influencing factors.

Key Words: Expression, hepatitis B virus, influencing factors, ovum, replication

Hepatitis B virus (HBV) infection is the most common chronic viral infection in the world.[1,2] The most prevalent mode of HBV transmission is vertical transmission. Vertical transmission includes intrauterine transmission, intrapartum infection, and puerperal infection.[3] Now HBV vertical transmission can be blocked by the use of antiviral drugs in late pregnancy and infants, in combination with immunoprophylaxis.[4-6] However, 5%–10% of transmission cannot be blocked because of intrauterine transmission.[7] Some researchers suspect that HBV is possibly transmitted vertically by germ cells, especially the ovum before pregnancy. Previous studies have found that HBsAg, HBcAg, and HBV DNA exist in the oocytes at different developmental stages in patients with chronic HBV infection (CHB).[8-10] They concluded HBV infection of the ovum occurred before vertical transmission of HBV. Yu *et al.* showed HBsAg expression in cells of the ovarian follicle or placental capillary endothelium signaling a higher risk for intrauterine HBV infection.[11] But what are the factors that lead to the infection of partial ovum? Some clinical observations prompted that HBeAg and HBV DNA levels in the serum of HBV infected mothers may be correlated with vertical transmission.[12,13] However, no study has explored the factors that influence the infection rate of the ovum and followed-up on clinical outcomes of vertical transmission.

In our study, we further clarified whether HBV can replicate in the ovum. In addition, we also analyzed the correlation between serum HBeAg level, HBV DNA level, and ovum infection with HBV by detecting the distributions of HBcAg, HBV DNA, and HBV mRNA that represent the HBV replication in ovarian tissues and ova of patients with CHB HBV.

MATERIALS AND METHODS

Subjects

Ovarian tissues were obtained from 50 patients with gynecological diseases and chronic HBV infection. Surgical removal of ovarian tissues were performed at the First Affiliated Hospital of Xi'an Jiaotong University and Shaanxi Maternal and Child Care Service Centre from July 2006 to June 2010. The average age of patients was 38 years. Gynecological diseases were as follows: 18 cases of ovarian cysts, two cases of ectopic pregnancy, six cases of ovarian teratoma and 24 cases of uterine cancer. Six cases of resected ovarian tissues without HBV infection were selected as negative controls. Liver tissues from two CHB patients were selected as positive controls. Patients with hepatitis A virus (HAV), hepatitis C virus (HCV), hepatitis D virus (HDV), and human immunodeficiency virus (HIV) infection were excluded.

Specimen treatment

Samples were first cut into sections of 1.5 cm × 1.2 cm with a thickness of no more than 0.2 cm and then quickly washed 8 times with PBS/DEPC. The sections were placed in 4% paraformaldehyde solution at 4°C for 4 h, followed by gradient alcohol dehydration and vitrification by xylene, and then the sections were embedded in paraffin. Samples with no HBV DNA detected in the final washing fluid were used in subsequent experiments. Paraffin sections were stretched to 4 μm in distilled water at 55°C without any additives and collected on bio-adhesive-coated glass slides. The slides were baked at 56°C – 60°C in a dry air oven for 4–6 h.

Immunhistochemical staining

Formalin-fixed and paraffin-embedded sections were deparaffinized in xylene and passed through an ethanol series. The sections were rinsed in 0.01 mol/L PBS. Nonspecific binding was blocked by treatment with 5% normal serum for 30 min and incubated with primary antibody overnight at 4°C (Rabbit anti-HBcAg, Abcam, Cambridge, United Kingdom). After secondary antibody (Goat antirabbit antibody, Abcam, Cambridge, United Kingdom) was applied and sections were incubated for 30 min at 37°C, they were incubated with avidin–biotin–peroxidase complex for 30 min at 37°C and washed again. The chromogen, 3-3-diaminobenzidine (DAB) was added onto the section for 10 min.

HBV DNA detection

Tissues were digested in 10 μg/mL of proteinase K in 0.l M Tris–EDTA buffer (pH 7.5) at 37°C for 20 min. Hybridization was performed for 18 h at 37°C with 10 μg/mL of a HBV DNA probe per section. The probe was labeled with digoxigenin at the thymine positions (Bioprobe Systems, TBD, Australia). For immunodetection, sections were washed in Tris–EDTA NaCl and incubated for 30 min at room temperature in a blocking solution in TEN-buffer, containing 6% dry skimmed milk. Mouse monoclonal antibodies were added to the sections by indirect immunohistochemical staining technique. They were incubated with avidin–biotin–peroxidase complex for 40 min at 37°C and washed again. The sections were incubated for 15 min after DAB was added.

Control groups were as follows: (1) Sections of other tissues (lymph node); (2) sections treated with DNase; (3) sections incubated with an excess of unlabeled HBV probe before hybridization; (4) sections of HBsAg-positive liver tissue as positive controls; (5) sections of ovaries from HBsAg-negative women as negative controls. Brown yellow coloration in the cytoplasm, cytomembrane, or nucleus was regarded as positive.

mRNA detection

All instruments and test tubes were soaked for 4 h with DEPC water. The RISH and AP Detection Kit (Cybrdi, USA) was used for the HBV-mRNA *in situ* hybridization. The probe was 5′-ACCATGTGGGCGTTCACGGTGGTCTCCATGC GACGTGCAGAGGTGAAGCGAAGTG-3′, the X region of HBV.

In brief, paraffin-embedded sections were dewaxed, digested, and dehydrated. Then were rinsed in 2× standard saline citrate (SSC) and dehydrated in ethanol followed by 20 μL of hybridization mixture consisting of 50% deionized formamide, 0.1 mol/L phosphate buffer (pH 7.0), 4× SSC, yeast tRNA (500 mg/mL), and 10% dextran sulfate, and 10 ng of heat-denatured-labeled probe was placed on each section. After hybridization, sections were washed for 1 h at 50°C with 3× SSC, digested with RNase A (20 mg/mL), and rinsed in 1.5× SSC and 0.75 × SSC at 50°C for 1 h. The digoxigenin-labeled hybrids were detected with a digoxigenin antibody–alkaline phosphatase conjugate and an enzyme substrate chromogen (nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate). Dark purple coloration in the cytoplasm or cytomembrane, or red coloration in the nucleus was regarded as positive.

Statistical analysis

Statistical analysis was performed using SPSS13.0 software (version 16.0; SPSS Inc., Chicago, IL, USA). Comparisons of categorical data were performed using McNemar test, with $P < 0.05$ considered as statistically significant. Group identification was performed using the Mann–Whitney U test.

RESULTS

Location of HBcAg and HBV DNA in ovary

Brown positive signals of HBcAg were detected in 6 ovarian tissues (12%, 6/50). HBcAg located within cytoplasm

and nucleus of the ova and granulosa cells [Figure 1]. HBV DNA was examined by *in situ* hybridization. Brown positive signals were detected in the interstitial cells, granulosa cells, and ova in ovarian tissues at a positive rate of 14% (7/50) [Figure 2]. No positive signal was observed in the blank or negative controls. There was a positive signal in the positive controls.

Distribution of HBV mRNA

To assess the infection and replication of HBV in samples, we examined HBV mRNA by *in situ* hybridization. Three ovarian tissues were positive for HBV mRNA (3%). Positive signal of HBV mRNA was mainly distributed in the cytoplasm of the ova and the granulosa cells. Only in one case, the positive signal was also observed in the primary ova in ovarian tissues at a positive rate of 2%. There was a positive signal in the positive controls, whereas the negative controls and blank controls were negative [Figure 3].

The relationship between HBeAg and HBV markers

To evaluate whether the infection status of HBV patients can influence HBV expression and replication in the ovum,
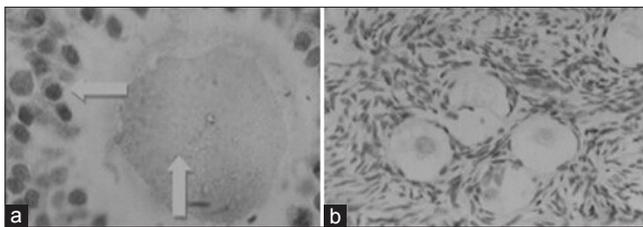

Figure 1: (a) Location of HBcAg in plasma, membrane, and nuclei of mature ovum (⇧), granular cell (⇐) (Original magnification: 10 × 100) (b) HBcAg-negative control (Original magnification: 10 × 40)
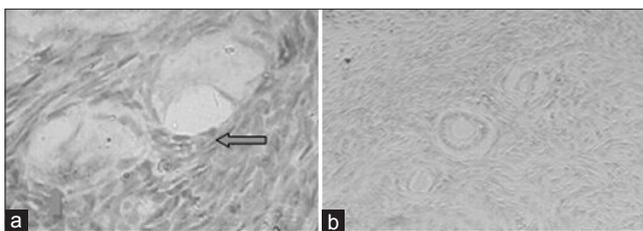

Figure 3: (a) Location of HBV-mRNA in plasma of primary ovum (⇧), granular cell (⇐) (Original magnification: 10 × 100) (b) HBV-mRNA-negative control (Original magnification: 10 × 40)

we compared the positive rate of HBcAg, HBV DNA, and HBV mRNA in ovaries between the serum HBeAg-positive group and serum HBeAg-negative group. The positive rate was significantly higher for HBeAg-positive women than HBeAg-negative ($\chi^2 = 13.14, P < 0.05; \chi^2 = 11.13, P < 0.05; \chi^2 = 17.39, P < 0.05$) [Table 1].

Positive rate of HBeAg for patients detected HBV markers in ovaria (HBcAg, HBV DNA, HBV mRNA respectively) were higher than HBeAg-negative [Figure 4].

Serum HBV DNA level and HBV markers

Samples were divided into low replication and high replication groups according to the sub-boundary of HBV DNA levels at 10^4 IU/mL. The positive rates of HBcAg, HBV DNA, and HBV mRNA in ovaries of high replication group were higher than those of the low replication
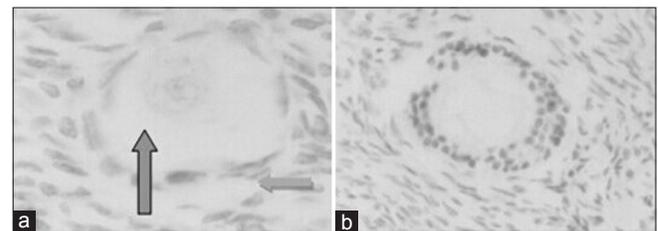

Figure 2: (a) Location of HBV DNA in plasma and nuclei of primary ovum (⇧), granular cell (⇐) (Original magnification: 10 × 100) (b) HBV DNA-negative control (Original magnification: 10 × 100)
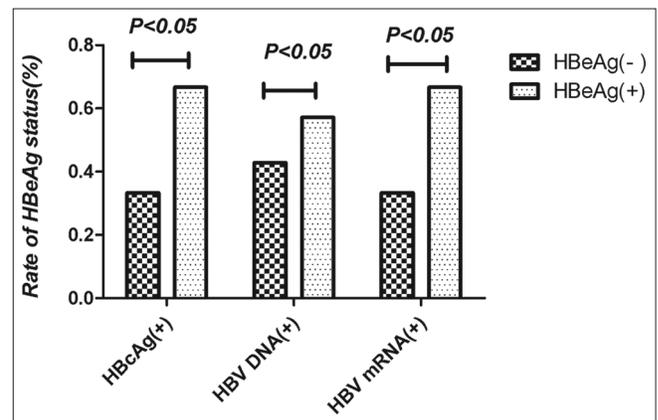

Figure 4: HBeAg status of patients detected with HBV markers in the ovary

Table 1: The relationship among serum HBeAg and positive signals of HBcAg, HBV DNA, and HBV mRNA in ovarium									
HBeAg	HBcAg in ovaries*			HBV DNA in ovaries**			HBV mRNA in ovaries***		
	Positive	Negative	Positive rate (%)	Positive	Negative	Positive rate (%)	Positive	Negative	Positive rate (%)
HBeAg+	4	20	16.7	4	20	16.7	2	22	8.3
HBeAg−	2	24	7.7	3	23	11.5	1	25	3.8
Total	6	44	12	7	43	14	3	47	6

McNemar test was used for analysis. *Shows the relationship between HBcAg and serum HbeAg: χ^2=113.14, *P*<0.05. **Shows the relationship between HBV DNA and serum HBeAg: χ^2=11.13, *P*<0.05. ***Shows the relationship between HBV mRNA and serum HBeAg: χ^2=17.39, *P*<0.05

group ($\chi^2 = 15.04$, $P < 0.05$; $\chi^2 = 12.96$, $P < 0.05$; $\chi^2 = 19.36$, $P < 0.05$, respectively) [Table 2].

Patients with detectable HBV markers in ovaries had a higher level of serum HBV DNA [Figure 5]. The mean logarithm HBV DNA level of ovary HBcAg-positive women in the study was 7.02 $\log_{10}$ IU/mL, compared with 4.46 $\log_{10}$ IU/mL in ovary HBcAg-negative women ($P < 0.05$). There were no significant differences in ovary HBV DNA and HBV mRNA positive group (5.87 $\log_{10}$ IU/mL vs 4.02 $\log_{10}$ IU/mL, $P = 0.71$; 6.52 $\log_{10}$ IU/mL vs 4.37 $\log_{10}$ IU/mL, $P = 0.64$).

Two out of the 50 patients had a positive signal of HBcAg, HBV DNA, HBV mRNA in ovarian tissue. Both were HBeAg positive and the viral load was much higher than 10^4 IU/mL (3.78×10^7 IU/ml and 1.19×10^8 IU/ml, respectively).

DISCUSSION

HBV enter into the host cell forming covalently closed circular DNA (cccDNA) together with HBV DNA polymerase. cccDNA is catalyzed by the host cell RNA polymerase and reverse transcribed into HBV mRNA and RT template and translation template.[14] HBV DNA and HBV mRNA are important components of HBV replication and expression. HBcAg is synthesized in the cytoplasm. After entering the nucleus, HBcAg is immediately assembled as HBV DNA, which is highly expressed and closely related to active viral replication.[15]

Previous studies found that HBV could infect the ovum[9,16-18] and speculated that HBV could enter embryos in this way and vertical transmission occurred via the ovum. In this study, HBV DNA, HBV mRNA, and HBcAg were detected in the ovum and ovarian tissues of patients with CHB. This observation provides evidence at the level of DNA, RNA, and protein supporting the hypothesis that HBV had replication and expression activity in ovarian tissues, especially the ova. Our results demonstrated that ova infected with HBV could transmit HBV via replication activity to the embryo after fertilization, and vertical transmission occurs at an early stage of life.

Both HBV serum markers and HBV DNA were detected as negative in the final wash fluid. The positive rate of HBV

Table 2: The relationship among serum HBV DNA levels and positive signals of HBcAg, HBV DNA, and HBV mRNA in ovarium

HBV DNA (IU/ml)	HBcAg in ovaries*			HBV DNA in ovaries**			HBV mRNA in ovaries***		
	Positive	Negative	Positive rate (%)	Positive	Negative	Positive rate (%)	Positive	Negative	Positive rate (%)
≤104	2	20	9.1	3	21	12.5	1	23	4.2
>104	4	22	15.4	4	22	15.4	2	24	7.7
Total	6	44	12	7	43	14	3	47	6.0

McNemar test was used for analysis. *Shows the relationship between HBcAg and the level of serum HBV DNA: χ^2=15.04, *P*<0.05. **Shows the relationship between HBV DNA and the level of serum HBV DNA: χ^2=12.96, *P*<0.05. ***Shows the relationship between HBV mRNA and the level of serum HBV DNA: χ^2=19.36, *P*<0.05
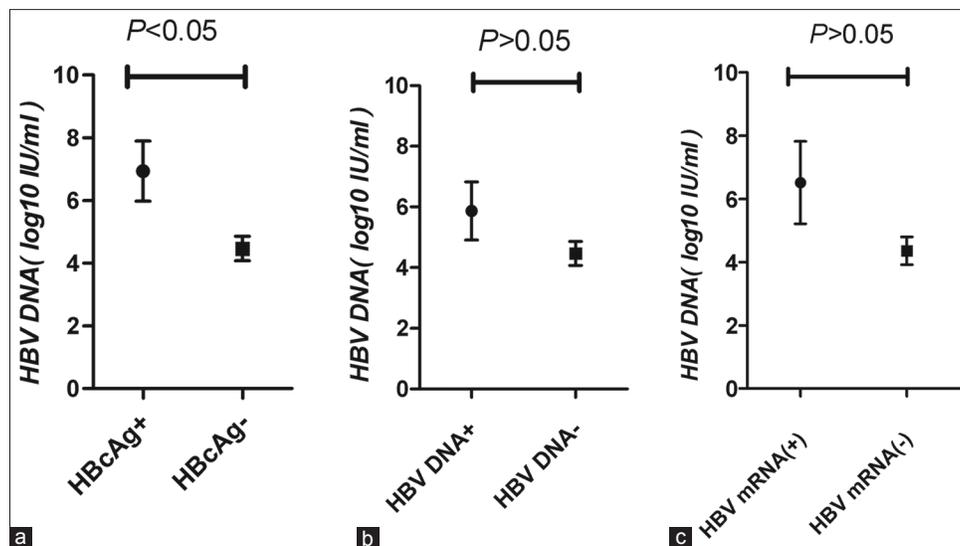

Figure 5: Serum HBV DNA of patients detected positive HBV markers in the ovary (a) Serum HBV DNA of patients detected positive HBcAg and negative in oocyte and ovarian tissues (b) serum HBV DNA of patients detected positive HBV DNA and negative in oocyte and ovarian tissues (c) serum HBV DNA of patients detected positive HBV mRNA and negative in oocyte and ovarian tissues

218

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mRNA is lower than that of HBV DNA in ovarian tissues. We infer that most of the HBV DNA sequences integrate into the host gene, but only a tiny part of the free existence show activity. On the other hand, HBV mRNA could not be displayed completely in every developmental stage of the ovum because the specimen we used in the study was only a fraction of ovarian cortex.

Many factors influence HBV vertical transmission. High levels of maternal HBV DNA is the main cause.[19,20] The conclusions were obtained from clinical observations. However, studies examining the mechanism are sparse. We first explored factors that influence the infection and replication of HBV in the ovum. We investigated the relationship among serum HBeAg status, HBV DNA levels, and ovum infection. HBcAg, HBV DNA, and HBV mRNA were detected in ovaries to verify infection and replication activity of HBV. Our results showed that HBV markers in ovaries were significantly higher in women with serum HBeAg-positive and/or high HBV DNA levels. Therefore, maternal viral load and immune state are important risk factors. This is consistent with most clinical observations. Treatment would be administered to these mothers for the purpose of suppressing the maternal viral load, thus minimizing risk of transmission.

This study demonstrated that serum HBeAg status and HBV DNA levels could influence HBV expression and replication in the ovum. Our findings provide the opportunity for the clinical practice of blocking vertical transmission of HBV. Another larger study is underway to clarify the mechanisms and risk factors of HBV-infected embryos.

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Conflicts of interest

All authors have no conflicts of interest regarding this paper.

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