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## The presence of HBV mRNA in the fertilized *in vitro* embryo of HBV patients confirms vertical transmission of HBV via the ovum

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### SUMMARY

This study aimed to confirm that vertical transmission of hepatitis B virus (HBV) can occur via the infected ovum. Specimens studied were obtained from discarded test-tube embryos from mothers with chronic HBV infection who had received *in vitro* fertilization treatment. Single-cell reverse transcriptase–polymerase chain reaction was used to detect HBV mRNA in the embryos. HBV mRNA was detected in the cleavage embryos of patients with chronic HBV infection, with a detection rate of 13·2% (5/38). The level of serum HBV DNA was not related to the HBV mRNA positivity rates in embryos. In this study, HBV mRNA was detected in test-tube embryos from HBV-infected mothers who had received *in vitro* fertilization treatment. This confirms the theory of vertical transmission of HBV via the ovum, thereby providing an important theoretical basis for further study on the mechanism of HBV vertical transmission, influencing factors and blocking measures.

**Key words:** Embryo, hepatitis B, ovum, vertical transmission.

### INTRODUCTION

Hepatitis B virus (HBV) can infect and replicate within the ovum [1], but vertical transmission of HBV via the ovum is a theory that has not been fully confirmed. To fully confirm this means of transmission involves resolving a number of questions, e.g. whether an HBV-infected ovum can be fertilized, whether an HBV-infected ovum can transfer HBV to the embryo, and whether HBV can continue to replicate in the

embryo thereby facilitating clinically significant vertical transmission of HBV. A number of difficult technical issues are involved, e.g. *in vitro* culture of the ovum, *in vitro* infection of the ovum, *in vitro* fertilization (IVF) of infected ova, etc., there are even some ethical issues involved. Therefore, it is difficult to confirm vertical transmission of HBV via the ovum with conventional research methods. In order to confirm this theory, we sought to detect HBV mRNA in a specimen of discarded test-tube embryos from HBV-infected mothers who had received IVF treatment. If we could detect HBV mRNA in a discarded embryo (the provider of sperm had no HBV infection, and the IVF and embryo culture process was conducted under strict conditions), the following points

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could be confirmed: (1) the ovum of a HBV-infected mother was infected with HBV and had replication activity; (2) this ovum can be fertilized successfully and transmit HBV to the embryo; (3) HBV in the embryo has replication activity. The above three points fully confirm that HBV is able to infect the ovum and that clinically significant vertical transmission of HBV has occurred.

## SUBJECTS AND METHODS

### Subjects

Thirty-eight discarded single-cell cleavage embryos were obtained from 14 couples who had received assisted reproductive technology treatment at the Reproductive Centre of Shaanxi Maternal and Child Care Service Centre from February 2007 to December 2008. Of these 14 couples, all the women had chronic HBV infection and the men had no infection. In the same period, 84 discarded single-cell cleavage embryos were obtained from 50 IVF couples with negative HBV markers for both men and women. These were taken as negative controls. Consent was obtained from the patients and the hospital ethics committee before the embryos were obtained, in accordance with the Declaration of Helsinki.

Ten millilitres of venous blood were collected from each patient before surgery and this was used for the quantitative detection of serum HBV DNA and the detection of hepatitis B serum markers. Meanwhile, HAV, HCV, HDV, and HIV antibodies were tested for in the serum of both husband and wife to rule out these viral infections.

In the ovum retrieval process, blood contamination was avoided as far as possible and a thorough cleaning was conducted; HBV serum markers and HBV DNA were detected as negative in the final wash fluid.

### Methods

Single-cell reverse transcriptase–polymerase chain reaction (RT–PCR) was the major test technique in this experiment.

#### Cell lysis

The discarded single-cell cleavage embryos were washed eight times with PBS/DEPC and then placed in sub-packaged lysate for 2 h of lysis. The final

Table 1. *Reverse transcription reagents*

Reagents	Volume
10 × buffer	2 $\mu$ l
25 mM MgCl <sub>2</sub>	4 $\mu$ l
0.1 M DTT	2 $\mu$ l
RNase out	1 $\mu$ l
Super III RT	1 $\mu$ l

wash fluid was retained for the detection of HBV DNA, and the specimens with negative HBV DNA in the final wash were used for subsequent experiments [2, 3].

#### Reverse transcription of RNA into cDNA

The reverse transcription reaction was conducted using a reverse transcription kit (Superscript III First-Strand Synthesis; Invitrogen, USA) according to the manufacturer's instructions, and the whole reaction was performed on ice. *Denaturation*: the reaction system is given in Table 1. The reaction mixture was mixed and centrifuged at 12 000 *g* for 3–5 s. Then after the mixture was incubated at 65 °C for 5 min, it was quickly placed on ice for at least 1 min and centrifuged at 12 000 *g* for 3–5 s. *Extension*: cDNA was synthesized. The reagents shown in Table 1 were added into the mixture one by one and gently shaken. When the total volume of the reaction system was 20  $\mu$ l, it was centrifuged at 12 000 *g* for 3–5 s, followed by a water bath at 25 °C for 10 min, and a further water bath at 50 °C for 50 min. *Termination*: the mixture was incubated at 85 °C for 5 min, and cooled on ice to terminate the reaction. After instantaneous centrifugation, 1  $\mu$ l RNase H was added to the mixture which was kept at 37 °C for 20 min to decompose untranscribed RNA and then stored at –20 °C or used immediately.

#### Amplification of housekeeping genes

*Primer sequences and characteristics*: the primers (Table 2) of housekeeping gene  $\beta$ -actin were designed with Oligo 6.0 biological software [4].

#### Amplification of HBV DNA by nested PCR

*Primer sequences and characteristics*: a pair of primers was designed according to mRNA gene sequences of the S area of HBV using Oligo 6.0 biological software (Table 3) [5]. The primers were P1, P3 (upstream) and P2, P4 (downstream).

Table 2. *Primer sequences and characteristics*

$\beta$ -actin	Primers' sequences (5'-3') (bp)	Amplification length
P1	5'-ATCGTGCGTGACATTAAGGAGAAG-3'	179 bp
P2	5'-AGGAAGGAAGGCTGGAAGAGTG-3'	

Table 3. *Primer sequences and characteristics*

HBV S area		Nucleic acid position	Primer sequences (5'-3') (bp)	Amplification length
Outer primers	P1	300-321	5'-CATCTTCTTGTTGGTTCTTCTG-3'	417 bp
	P2	715-695	5'-TTAGGGTTTAAATGTATACCC-3'	
Inner primers	P3	421-441	5'-TCTATGTTTCCCTCTTGTTC-3'	206 bp
	P4	626-605	5'-TACCACATCATCCATATAACTG-3'	

#### *Electrophoresis of PCR end products on 1.5% agarose gel*

The prepared 1.5% agarose gel was heated and melted in a microwave oven and after cooling to 60 °C, it was poured into a gel mould with a preset electrophoresis comb. After 30-45 min at room temperature, the electrophoresis comb was removed and the gel was placed into an electrophoresis tank and  $1 \times$  TBE electrophoresis buffer added. A 10- $\mu$ l real-time quantitative PCR product of each gene was taken from each group, loading buffer was then added in order to display DNA and make the sample sink to the bottom. Voltage electrophoresis at 80 V was conducted for 35 min and electrophoresis fragments were observed under ultraviolet light. An imaging system (Bio-Rad, USA) was employed for scanning and the images were printed.

#### *Controls*

*Negative controls:* (a) the discarded embryos from the couples with negative HBV serum markers for both man and woman; (b) no template was added in the reverse transcription process; (c) no reverse transcriptase was added in the reverse transcription process; (d) no template was added in the PCR reaction system.

*Positive control:* HBV patients with positive serum DNA.

#### *Follow-up visits*

The infants with HBV-infected mothers were followed up every 3 months. The infants' peripheral blood was obtained for the detection of HBV serum markers. The follow-up visits lasted for 12 months.

## RESULTS

### RT-PCR results

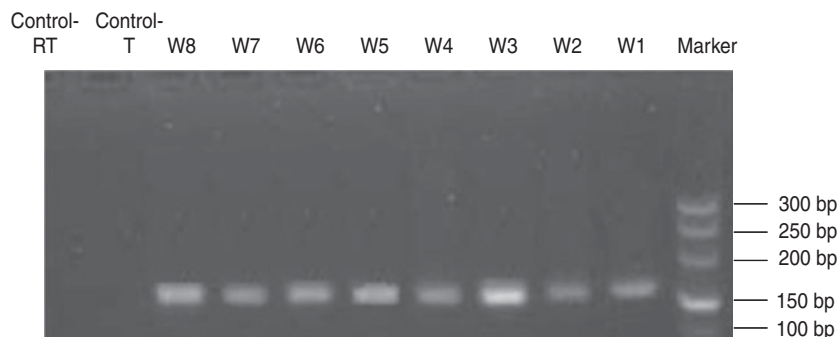
All single-cell cleavage embryos were placed in lysate and reverse transcription was performed to generate cDNA in the same Eppendorf tube. The housekeeping gene  $\beta$ -actin was amplified by PCR with an amplified fragment length of 179 bp. Except for the negative controls, the target fragments were amplified in every tube (Fig. 1).

The target fragments were amplified by nested PCR and the products were 206 bp in the second round (Fig. 2). The positive rate of cleavage embryos was 13.2% (5/38). These five positive embryos were from two cases of the 14 couples; in one case, three positive signals were detected in five embryos with serum HBeAg positive and HBV DNA quantification at  $4.7 \times 10^5$  IU/ml; in the other case, two positive signals were detected in the three cleavage embryos with serum HBeAg negative and HBV DNA quantification  $\leq 10^3$  IU/ml. No specific HBV mRNA signal was detected in the embryos of the negative controls.

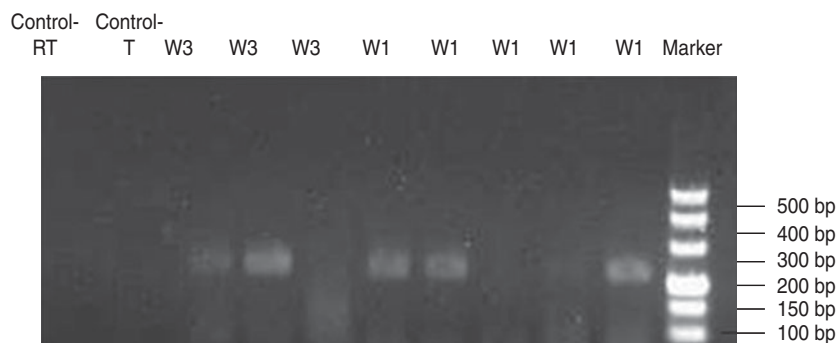
The target fragments were amplified by nested PCR, and there was no amplified fragment in the first round, the product was 206 bp in the second round. In the HBV-positive mother group, for one case three positive signals were detected in five embryos and in the other case two positive signals were detected in three cleavage embryos.

### Follow-up results

After follow-up visits, it was found that of the two pregnant women from the couples with the mother



**Fig. 1.** Electrophoresis of  $\beta$ -actin. With the exception of negative control the target fragments were amplified in each tube. The length of the amplified fragment was 179 bp.



**Fig. 2.** Electrophoresis of HBV mRNA PCR products in a single cleavage embryo. The target fragments were amplified by nested PCR and the product was 206 bp. No specific HBV mRNA signal was detected in the embryos of the negative control. These five positive embryos were from two cases of the 14 couples (W1 and W3).

infected with hepatitis B and HBV RNA detected in cleavage embryos, one woman was successfully pregnant and hepatitis B was negative in this woman's infant at both birth and at age 6 months.

## DISCUSSION

HBV is capable of infecting oocytes [1] and replicating therein. On this basis, the hypothesis that vertical transmission of HBV occurs via the ovum is put forward. Furthermore, some scholars have transfected the HBV gene into a mouse ovum which was then fertilized with normal sperm, or directly transfected the HBV gene into a fertilized ovum. In these mice not only were integrated HBV DNA fragments [6, 7] found, there was also evidence of HBV replication and expression [8], and even 3.5 kb mRNA and a small amount of double-stranded DNA packaged in Dane particles was found [9, 10]. These studies show that after transfection, HBV could enter the embryos via the animal's ovum and replicate in them, and could even mature in the mother's body and generate new individuals. However, HBV has strict species specificity, and a large gap exists between the

transgene process and natural infection; therefore there is a great difference between the above research and human HBV vertical transmission via the ovum in the natural process. Human oocytes are not readily available, therefore research on confirmation of vertical transmission of HBV via the ovum is very slow.

In this study, HBV mRNA was detected for the first time in early human embryos, which suggests that HBV in these embryos has a certain replication activity and is capable of being transcribed and translated under certain conditions. All the patients in the study were receiving IVF treatment and blood contamination was avoided as far as possible in the ovum retrieval process and a thorough cleaning was conducted; both HBV serum markers and HBV DNA were detected as negative in the final wash fluid. The sperm providers were not HBV-infected individuals, and the IVF process was conducted under very strict conditions where no contamination factors existed. Therefore, the presence of HBV in the embryos could only have come from the ovum and the HBV that infected the ovum was able to enter the embryos during the process of ovum fertilization and then

replicate actively within; vertical transmission of HBV then occurs via the ovum in the real sense.

The infant of the only successfully pregnant mother with HBV mRNA detected in the embryo was followed up, and no HBV evidence was detected in the infant's serum at birth or at age 6 months. This result is consistent with previous experimental results on HBV-infected ova, which suggests that not all ova of patients with chronic HBV infection can be infected with HBV. Moreover, it was also found in this study that only part of the cleavage embryo of the same patient with chronic HBV infection was infected with HBV, which further confirms the above result.

This study found that HBV patients' embryos infected with HBV was not correlated with viral load, which indicates that transmission of HBV via the ovum is not correlated with quantitative level of virus. At present, a blocking measure, i.e. lowering the serum HBV viral load of HBV patients, is not effective in this transmission via the ovum. The mechanism may be related to the person carrying virus, which needs further study.

In this study, HBV mRNA was detected in discarded test-tube embryos from HBV-infected mothers after IVF treatment, which confirms clearly for the first time the theory of vertical transmission of HBV via the ovum. This provides an important theoretical basis for further study on the mechanism of vertical transmission of HBV, influencing factors and blocking measures.

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#### DECLARATION OF INTEREST

None.

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