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The effect of hepatitis B virus infected embryos on pregnancy outcome

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ABSTRACT

Objective: HBV vertical transmission is the main reason for chronic HBV infection, but there is no clear conclusion about the effect of HBV infection on pregnancy outcome. This study aimed to investigate the effect of HBV-infected embryos on pregnancy outcome.
Study design: 75 couples who received ART treatment were followed up. Among them were 25 couples (group A) with at least the husband or wife infected with HBV, and 50 couples (group B) with negative serum HBV markers of both husband and wife. The clinical pregnancy rate, early abortion rate, neonatal malformation rate, neonatal HBV infection rate, and HBV infection rate of the six-month-old infant were compared between these two groups, and Fisher's exact test was applied. HBV mRNA in abandoned embryos of the patients was detected by single-cell RT-PCR.
Results: Both the early abortion rate and the abortion rate with the mother infected HBV in group A were significantly higher than in group B ($P = 0.043$, $P = 0.030$ respectively). The clinical pregnancy rates of groups A and B were 36% and 44% respectively ($P = 0.248 > 0.05$). The neonatal malformation rate, neonatal HBV infection rate, and HBV infection rate of six-month-old infants in group A and group B were all 0. Specific HBV mRNA fragments were detected in 6 cases of 62 cleavage embryos in group A, giving a positive rate of 9.7%. The positive rates with mother infected and father infected with HBV were 13.2% (5/38) and 5.6% (1/18) respectively.
Conclusions: HBV infection can increase the early abortion rate of pregnancy, and the reason may be related to HBV-infected embryos.

1. Introduction

Hepatitis B virus (HBV) is a worldwide disease that seriously harms human health. Currently about 2 billion people around the world have been previously or persistently infected with HBV, among whom 350–400 million people are patients with chronic HBV infection [1]. HBV vertical transmission is the main reason for chronic HBV infection, but there is no clear conclusion about the effect of HBV infection on pregnancy outcome. As a virus, HBV may theoretically have an adverse effect on pregnancy and the development of embryos. Studies by Safir et al. suggest that HBV-infected patients have higher rate of miscarriage and preterm birth after pregnancy, and have more pregnancy complications [2–4], but some investigators found that HBV infection has no effect on the embryo fertilization rate, cleavage rate, high quality embryo rate, clinical pregnancy rate or early abortion rate [5,6].

It was found in a study on HBV vertical transmission by our research group that the pregnancy outcome of HBV-infected patients was different from that of non-infected people, and further research found that HBV infection might be the main reason for high miscarriage rate.

2. Materials and methods

2.1. Subjects

Sixty-two abandoned embryos were selected as research subjects from 25 couples who had received assisted reproductive technology treatment in the Reproductive Center of Shaanxi Maternal and Child Health Hospital from February, 2007 to December, 2008 (group A). Among these 25 couples, 14 couples (wife with chronic HBV infection and husband with HBV serological markers negative) and their 38 abandoned single cell

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cleavage embryos were designated as group A1, 9 couples (husband with chronic HBV infection and wife with HBV serological markers negative) and their 18 abandoned embryos were group A2, and 2 couples (both husband and wife with chronic HBV infection) and their 6 abandoned embryos were group A3. Eighty-four abandoned embryos were selected as a negative control (group B) from 50 couples (both husband and wife with HBV serological markers negative) who had received ART concurrently. Consent was obtained both from the patients and from the Ethics Committee of the hospital before the embryos were obtained, and this was in accordance with the Declaration of Helsinki. All HBV-infected patients were diagnosed with chronic hepatitis B or as chronic HBV carriers, which was in accordance with the standards of the 2010 Chinese chronic hepatitis B prevention and treatment guidelines [1].

Preoperative serum samples of the couples were obtained and detected to exclude hepatitis A, C and D infection and HIV infection, and quantitative detection of HBVDNA and the detection of HBV serum markers were conducted.

2.2. Experimental methods and steps

2.2.1. Follow-up

People included in the study were followed up and data were collected to compare the clinical pregnancy rate, early abortion rate, neonatal malformations rate, neonatal HBV infection rate, and baby HBV infection rate at half a year after the birth between group A and group B. Then statistical analysis was conducted.

2.3. HBV mRNA detection

Single-cell RT-PCR was used to detect HBV mRNA in the embryos [7]. The main reagents and instruments were: the SuperScript III First-Strand Synthesis System, Platinum Taq DNA polymerase (5u/μL), RNasin, dNTP (10 mmol/L), 10× buffer were purchased from Invitrogen Corporation, USA. Biometra gradient PCR amplification instrument was purchased from Whatman Biometra Corporation, Germany.

1. Cell lysis: Abandoned single-cell cleavage embryos was washed for 8 times with PBS/DEPC and then placed in repackaged lysis buffer (ice configuration, 40 μL 10× SuperScript III first-strand RT buffer, 2 μL NP-40Cells, 4 μL RNAsin, 54 μL DEPC water, 5 μL for each package and stored at −20 °C) and was lysed for two hours. The washing fluid of last time was used for the detection of HBV serum markers and HBVDNA. HBV serum markers and HBVDNA in the washing fluid of last time were detected to be negative, and then these specimens could be used for subsequent experiments.

2. RNA was reverse transcribed into cDNA: (1) According to the instructions of Invitrogen's Superscript III First-Strand Synthesis reverse transcription kit, reverse transcription reaction was conducted, and the whole reaction was carried out on ice. (2) Denaturation: the reaction system (6 μL total RNA, 1 μL Oligo (dT), 1 μL dNTP Mix, 2 μL DEPC water, 10 μL total reaction system, RNA concentration – 0.2 μg/μL) was mixed evenly and centrifuged at 12,000 × *g* for 3–5 s, and after it was incubated at 65 °C for 5 min, it was quickly placed on ice for at least 1 minute, and then centrifuged at 12,000 × *g* for 3–5 s. (3) Extension: the reaction system of cDNA synthesis, (2 μL 10× buffer, 4 μL 25mM$MgCL_2$, 2 μL 0.1 M DTT, 1 μL RNase out, 1 μL Super III RT) was gently shaken, and the total volume of the reaction system was 20 μL. The reaction system was centrifuged for 3–5 s, and 25 °C water bath was performed for 10 min and 50 °C water bath for 50 min. (4) Termination: The reaction system was incubated at 85 °C for 5 min and cooled on ice, and then the reaction was terminated. (5) After 12,000 × *g* transient centrifugation, 1 μL RNase H was added to the reaction system and the incubation at 37 °C lasted for 20 min, and non-transcribed RNA was decomposed. The system was stored at −20 °C or used directly.

3. Amplification of housekeeping gene: (1) The primer sequences and their characteristics: Oligo6.0 biological software was used to design the primer of housekeeper gene β-actin. The primer (P1 5′-ATCGTGCGTGACATTAAGGAGAAG-3′, P2 5′-AGGAAGGAAGGCTG-GAAGAGTG-3′, amplification length – 179 bp). (2) PCR reaction system (0.5 μL Taq DNA polymerase (5u/μL), 5 μL 5× buffer (1.5 mmol/L$MgCl_2$), 1 μL of P1 (100 μmol/l), 1 μL P2 (100 μmol/l), 0.5 μL dNTP (10 mmol/l), 10 μL template cDNA, 4 μL 50 mmol/L $MgCl_2$, 28 μL ddH_2O, and the total reaction system was 50 μL). (3) PCR reaction amplification conditions: predenaturation: 95 °C, 30 s; denaturation: 95 °C, 30 s; renaturation: 58 °C, 30 s; extension: 72 °C, 30 s for 35 cycles, and then 72 °C for 10 min.

4. Nested PCR amplification of HBVDNA: (1) The primer sequences and their characteristics According to mRNA gene sequences in S region of HBV, Oligo6.0 biological software was used to design a pair of primers (outer primer, P1 5′-CATCTTCTTGTTGGTTCTTCTG-3′, P2 5′-TTAGGGTTTAAATGTA-TACCC-3′, amplification length – 417 bp, P3 5′-TCTATGTTTCCCTCTTGTTGC-3′, P4 5′-TACCACATCATCCATA-TAACTG-3′, amplification length – 206 bp. Upstream primers: P1, P3; downstream primers; P2, P4) (2) The first round of PCR reaction system (0.5 μL Taq DNA polymerase (5u/μL), 5 μL 5× buffer (1.5 mmol/L$MgCl_2$), 1 μL P1 (100 μmol/l), 1 μL P2 (100 μmol/l), 0.5 μL dNTP (10 mmol/l), 10 μL template cDNA, 4 μL 50 mmol/L $MgCl_2$, 28 μL ddH_2O, and the total reaction system was 50 μL). (3) PCR reaction amplification conditions: predenaturation: 95 °C, 3 min; denaturation: 94 °C, 50 s; renaturation: 56 °C, 50 s; extension: 72 °C, 50 s, 30 cycles, extension at 72 °C for 10 min, and then the second round of PCR was conducted. (4) The second round of PCR reaction system (0.5 μL Taq DNA polymerase (5u/μL), 5 μL 5× buffer (1.5 mmol/L$MgCl_2$), 1 μL the P3 (100 μmol/l), 1 μL P4 (100 μmol/l), 0.5 μL dNTP (10 mmol/l), 2 μL template cDNA, 4 μL 50 mmol/L $MgCl_2$, 36 μL ddH_2O, and the total reaction system was 50 μL). (5) The amplification conditions were the same as that of the first round.

5. Final PCR fragment was subjected to electrophoresis on 1.5% agarose gel: Prepared 1.5% agarose gel was heated and melted in a microwave oven, and when it was cooled to 60 °C, it was poured into plastic mold of preset electrophoresis comb. After it was put under room temperature for 30–45 min, the electrophoresis comb was pulled out, and the gel was placed in the electrophoresis tank, and then 1× TBE electrophoresis buffer was added. Loading buffer was added to 10 μL real-time quantitative PCR product of each gene in each group. 80 V voltage electrophoresis was conducted for 35 min, and electrophoresis fragment was observed under ultraviolet light.

6. Assay for HBV serum markers and HBVDNA in the washing fluid: HBsAg and HBeAg in the medium were detected using an ELISA kit (Antigen de HBs, Basel, Switzerland) obtained from Roche. Detection of HBVDNA by Q-PCR: After extraction from the medium, HBVDNA was measured using a Q-PCR diagnostic kit from Da-An Gene Corp.

7. Control selection: Negative controls (a) abandoned embryos after ART with the couples' HBV serum markers negative; (b) reverse transcription process without template; (c) reverse transcription process without reverse transcriptase; (d) PCR reaction system without template. The positive control was HBV patients with positive serum DNA.

2.4. Statistical analysis

Data were analyzed using SPSS16.0, and Fisher's exact test was used to compare rates.