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Replication and Translation of Sperm-introduced Hepatitis B Virus Genes in Embryonic Cells

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ABSTRACT

Objective: Given that hepatitis B virus (HBV) can infect human sperm cells and HBV genes introduced by sperm can be transcribed in developing embryos, we sought to confirm the presence of mature HBV antigens and to assess the mechanism of viral gene replication in embryonic cells.

Methods: Human sperm were transfected with recombinant plasmids pIRES2-EGFP-HBs or pIRES2-EGFP-HBc and were then used to fertilize zona-free golden hamster ova. Two-celled embryos with and without green fluorescence were collected for detection of HBV DNA or protein by fluorescence in situ hybridization (FISH), immunofluorescence assay (IA), and enzyme-linked immunosorbent assay (ELISA).

Results: 1. Positive FISH signals for HBs- and HBc-DNA were observed in the sperm nuclei and in the nuclei of the two-celled embryos in the test samples but were not detected in the controls. 2. Positive IA signals for HBsAg or HBcAg were detected within the cytoplasm of the two-celled embryos in the test samples but not in the controls. 3. Using ELISA, the level of HBsAg in a single two-celled embryo was 0.064 ng/ml. Likewise, HBcAg was detected in the five-embryo experimental group.

Conclusion: The sperm-introduced HBV S or C genes were able to replicate concomitantly host embryo genome via a semiconservative mode of DNA replication and were able to express viral proteins in the embryonic cells.

INTRODUCTION

Hepatitis B virus (HBV) infection is a serious health problem worldwide. The World Health Organization (WHO) has estimated that around 500 000 000 people are chronically infected HBV or HCV. Approximately 1 000 000 people die each year (~2.7% of all deaths) from causes related to viral hepatitis, most commonly liver disease, including liver cancer [1]. The transmission routes of HBV through blood transfusion [2], body fluids (i.e., serum, saliva, vaginal secretions, breast milk, and semen) [3-6], intrauterine infection [6], cell, tissue and organ transplantation [7,8], and others mechanisms including hemodialysis units, intravenous drug injection and occupational exposure [9,10] have been documented. As the transmission routes of pathogenic viruses are identified,

the severity of the viral threat to human health can decrease as effective preventive measures are adopted. New, but unidentified, transmission routes pose a greater risk potential because even when viral reservoirs are known, current preventative actions may prove ineffective at blocking these new routes.

In his Nobel lecture of 1977, Dr. Blumberg suggested that HBV could enter the nucleus of its host and be transmitted vertically with the genetic material [11]. Subsequently, Hadchouel et al identified the presence of integrated HBV DNA sequences in spermatozoa from two of three patients with HBV infection [12]. Wang et al. examined the sera collected from 16 father/infant pairs where the fathers were known HBV carriers, while the mothers were negative for HBV markers. They detected 98-100% and 99-100% homology of the HBV surface or envelop S (nucleotides 451-660) and the HBV core C (nucleotides 2022-2301) gene sequences, respectively, between the father and infant [13,14]. It was hypothesized that the presence of the integrated sequences in spermatozoa may facilitate vertical transmission of HBV via the germ line, but to date no one has confirmed their assumptions. Two major questions must be addressed in testing this hypothesis. First, can human sperm harboring HBV genes successfully traverse the oolemma and enter the oocyte? Second, can these sperm-introduced HBV genes replicate and express viral mRNA and proteins in embryonic cells? Interspecies *in vitro* fertilization of zona-free golden hamster ova by human sperm has been widely used in the study of reproductive biology [15] and was previously employed to provide direct evidence that HBV DNA could integrate into the sperm chromosomes of patients and these human sperm carrying HBV genes could pass through the oolemma and result in normal fertilization [16]. After fertilization, the sperm-introduced HBV S and C genes were transcribed into viral mRNAs in embryonic cells [17]. However, it is not clear whether the sperm-introduced HBV S and C genes retain their competency in replication and protein expression in embryonic cells.

The viral envelope protein encoded by the HBV S gene results in the expression of three distinct isoforms, synthesized in all patients, termed the large, middle, and major proteins. Several antigenic determinants, including the “a” determinant, common to all Hepatitis B S antigens (HBsAg), as well as the “d”, “y”, “w”, and “r” determinants, are key epidemiologic factors. The HBV C gene encodes the Hepatitis B core antigen (HBcAg), which is the nucleocapsid protein that encapsulates the viral DNA. When HBcAg-derived peptides are expressed on the surface of hepatocytes, they induce a cellular immune response that is critical to the detection and killing of infected cells [18]. Therefore, the current study was undertaken to explore whether the sperm-introduced HBV S and C genes retain their capacity to replicate and express mature protein in embryonic cells.

MATERIALS AND METHODS

Ethical Approval

Semen samples were collected from healthy donors. Written informed consent was obtained from all subjects permitting the use of their sperm samples for research purposes, after being explicitly informed about the research aims and their rights. All protocols involving human and animal subjects used in this study were approved by the Ethics Committee of Shantou University Medical College (approval number: SUMC-00-0031) and by the Institutional Ethical Review Board of Chengdu Jinjiang Hospital for Maternal and Child Health Care (approval number: CJHMCHC-0010) according to the recommended guidelines of National Institute of Health involving human subjects and animal care and the 1975 Declaration of Helsinki.

Subjects

Three healthy men between 20 and 22 years of age were selected, who underwent routine physical examinations including laboratory tests, chest x-rays, EKGs, urinalysis and stool testing, and HEENT (head, eyes, ears, nose, and throat) evaluation. All three individuals were negative for HBV markers as assessed using an antigen-antibody combination assay and presented normal semen parameters according to the WHO laboratory manual.

Plasmid and Media

The recombinant plasmids pIRES2-EGFP-HBs and pIRES2-EGFP-HBc have been previously described [19]. Biggers-Whittingham (BWW) medium supplemented with 0.3% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO, USA) was prepared for human sperm preparation, oocyte collection, insemination, and subsequent handling. Ovum culture medium (OCM, Flow Laboratories, Germany) containing 10% heat-inactivated fetal bovine serum was used for post-insemination culture.

Preparations of Human Spermatozoa

Semen samples were obtained from healthy men without HBV infection and incubated in a CO₂ incubator (37 °C, 5% CO₂ in air) for 30 min to allow liquefaction. Motile spermatozoa were selected by the swim-up method and were collected by centrifugation at 600 x g for 5 min. Sperm were re-suspended in BWW medium with 0.3% BSA and centrifuged again. The washed spermatozoa were re-suspended in 5 ml of 10 μM ionophore solution for 8 min to facilitate capacitation, washed twice, and then suspended in BWW with 3.5% BSA to complete capacitation.

Exposure of Spermatozoa to Plasmids pIRES2-EGFP-HBV

After 2.5 h incubation the spermatozoa were transfected with the recombinant plasmids. Briefly, 100 μl of a mixture containing 1 μl pIRES2-EGFP-HBs (or pIRES2-EGFP-HBc) (1.5 μg/ml), 6 μl liposome, and 93 μl HEPES buffered saline were

incubated at room temperature for 15 min, subsequently added to the sperm, and then returned to the incubator for another 1.5 hr. Transfected sperm were then washed five times in 5 ml BWW medium.

Preparation of Golden Hamster Oocytes

Mature hamsters were induced to super-ovulate by intraperitoneal injection of 40 IU of pregnant mare serum gonadotropin (PMSG, Ningbo Hormone Product Co., Ltd., Ningbo, China), on day 1 of the estrous cycle. After 72 hours, the hamsters were injected, intraperitoneally with 40 IU human chorionic gonadotropin (hCG, Ningbo Hormone Product Co., Ltd.). Super-ovulated oocytes were collected from the ampulla region of the oviducts 17 hr after hCG injection and freed from the cumulus cells in 0.1% hyaluronidase (Sigma Chemical Co.). Cumulus-free oocytes were washed twice in BWW medium with 0.3% BSA, treated for 2 min with 0.1% trypsin (Sigma Chemical Co.) at room temperature to remove the zona pellucida, and then washed twice in BWW medium with 0.3% BSA.

Insemination and Post-insemination Culture

Insemination was performed using sperm suspensions at a concentration of 10^6 capacitated sperm/ml. Oocytes were kept in the sperm suspension for 20-30 min, then transferred to and incubated in BWW medium with 0.3% BSA under mineral oil (Sigma Chemical Co.) for another 40 min to ensure sperm penetration. After washing twice in OCM, groups containing five oocytes each, were cultured in a droplet (60 μ l each) of OCM under oil in a plastic Petri dish kept in a CO₂ incubator (37 °C, 5% CO₂ in air) for 24 hr.

Preparations of Embryos

Twenty-four hours after insemination, two-cell stage embryos expressing green fluorescent protein were selected under a fluorescence microscope (Axiovert 40 CFL, Zeiss, Germany), washed three times in cold 1X phosphate buffered saline (PBS) to remove serum from the medium, and used for FISH, immunofluorescence assay, or ELISA.

Labeling of HBV DNA Probe and FISH

Recombinant plasmids containing the HBs and HBc genomic DNA, respectively were used to amplify the respective HBV genes as previously described^[20]. Briefly, the HBs and HBc DNA were amplified, respectively, by PCR and purified using the Uniqu-10-PCR product purification kit (Saigon, Shanghai, China). The HBs- and HBc-DNA probes were labeled with biotin-14-dATP via nick translation (BioNick DNA Labeling System, GIBCO BRL, Waltham, MA, USA). Unincorporated nucleotides were removed by cold ethanol precipitation.

FISH and detection of hybridized probes were performed as previously described^[16] using a fluorescence microscope (DM2500, Leica, Germany) with WB excitation filters.

Immunofluorescence Assay

The two-celled embryos were fixed with 4% paraformaldehyde in PBS at room temperature for 15 min, and then treated with a mixture containing 0.5% Triton X-100, 0.5 mM MgCl₂, 5 mM EGTA, and 50 μ M glycine at room temperature for 15 min. Fixed embryos were placed in a blocking solution containing 2% fetal bovine serum and 10% normal goat serum in 0.1% PBS at 4 °C for 1 hr and then divided into three groups: group A and B were incubated with primary anti-HBsAg or anti-HBcAg antibodies overnight at 4 °C, respectively, and group C was incubated with PBS as the negative control. After washing three times in PBS for 5 min each, all the samples were incubated with biotin-labeled normal goat serum (1:100 dilution) at 37 °C for 1 hr, and then washed four times in PBS followed by propidium iodide (0.1 mg/ml) staining for 5-10 min. DABCO (1,4-Diazabicyclo [2.2.2] octane) was used as the anti-fade reagent.

ELISA

Assays to detect HBV protein were carried out according to the manufacture's instruction using an HBsAg ELISA Kit (InTec Products, Inc., Xiamen, China). The standard curve was generated using CurveExpert (version 1.3) and the HBsAg level was calculated according to the standard curve.

Two-celled embryos were separated into three groups, containing one, two, or five embryos, respectively. Following protein extraction, the assay to detect HBcAg was performed according to the manufacturer's instructions using an HBcAg ELISA Kit (Beijing Medical Biotech Co., Ltd., Beijing, China).

RESULTS

Plasmids pIRES2-EGFP-HBV and Two-Cell Embryos

Because the plasmid pIRES2-EGFP-HBs (or pIRES2-EGFP-HBc) contains the HBs gene (or the HBc gene) and an enhanced green fluorescent protein (EGFP) reporter construct for HBs (or HBc) gene expression, it was possible to distinguish the embryos with or without the HBs (or the HBc) gene by the presence of green fluorescence in embryos after fertilization of oocytes by sperm transfected with either the pIRES2-EGFP-HBs or the pIRES2-EGFP-HBc plasmid. A total of 62 fluorescing embryos were identified from 258 embryos collected, indicating an overall rate of efficiency of about 24%.

FISH

The hybridization signals for HBs or HBc DNA were detected in the nucleus of a portion of the spermatozoa transfected with the pIRES2-EGFP-HBs or pIRES2-EGFP-HBc plasmids (**Figure 1**). The hybridization signal for HBs or HBc DNA was likewise observed in both nuclei of the individual two-celled embryos exhibiting green fluorescence (**Figures 2 and 3**) but was not observed in the negative controls without detectable green fluorescence.

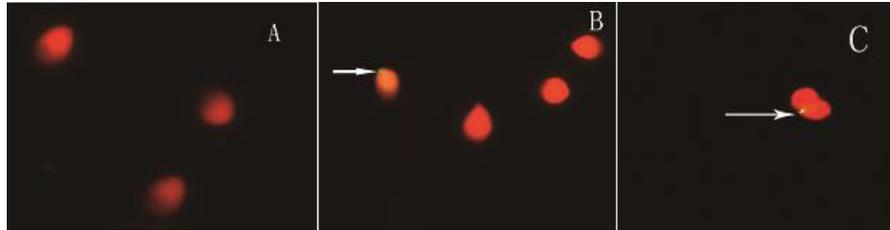


Figure 1. FISH results of human spermatozoa after transfection by recombinant plasmid pIRES2-EGFP-HBs or pIRES2-EGFP-HBc, x 1000. A: Negative control transfections show no HBV DNA signal. B: pIRES2-EGFP-HBs-transfected sperm show a positive signal for HBs DNA in the sperm head (arrow). C: pIRES2-EGFP-HBc-transfected sperm show a positive signal for HBc DNA in the sperm head (arrow). The results indicate that HBs and HBc DNA sequences have integrated into the sperm genome.

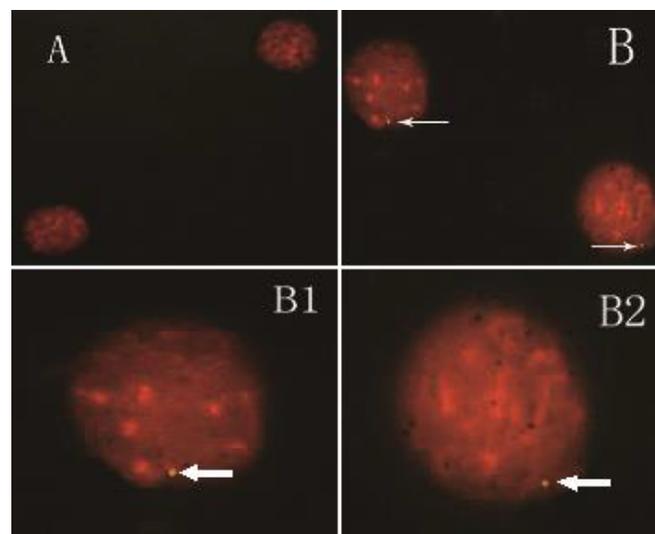


Figure 2. FISH results of two-celled embryos, x 1000. A: No signal for HBs DNA is detected within either nucleus of a two-celled embryo in the control sample. B: The positive signals for HBs DNA within each nucleus of a two-celled embryo in the experimental sample (arrows). B1 and B2: Increased magnification of each nucleus in part B, The results suggest that the replication of the sperm-introduced HBs gene is semiconservative and synchronized with that of the host genome, and the two viral gene copies segregate into the two daughter cells during the first cleavage.

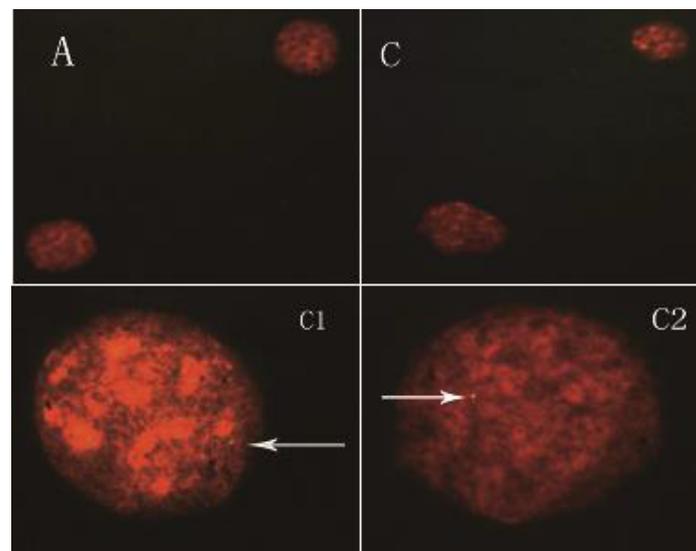


Figure 3. FISH result of two-celled embryos, x 1000. A: No signal for HBc DNA is detected within either nucleus of a two-celled embryo in the control sample. C: The positive signals for HBc DNA within each nucleus of a two-celled embryo in the experimental sample (arrows). C1 and C2: Increased magnification of each nucleus in part C. The results suggest that the replication of the sperm-introduced HBc gene is semiconservative and synchronized with that of the host genome, and the two viral gene copies segregate into the two daughter cells during the first cleavage.

Immunofluorescence Assay for HBsAg and HBcAg

The results of the immunofluorescence assay for HBsAg and HBcAg are shown in **Figures 4 and 5**. Clear HBsAg and HBcAg signals were detected within the cytoplasm of the embryonic cells from the respective experimental fertilization, while no positive signal was detected within the cytoplasm of the control embryonic cells.

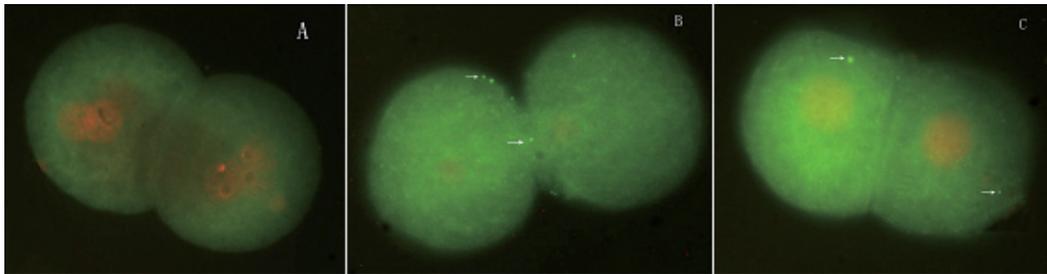


Figure 4. The results of the immunofluorescence assay, x 1000. A: No positive signal is detected within the cytoplasm of embryonic cells in the control sample. B: The positive signals for HBsAg are detected within the cytoplasm of the embryonic cells in the experimental sample (arrows). C: The positive signals for HBcAg are detected within the cytoplasm of the embryonic cells in the experimental sample (arrows). The results suggest that the sperm-introduced HBs and HBc genes are able to express HBsAg and HBcAg in the embryonic cells.

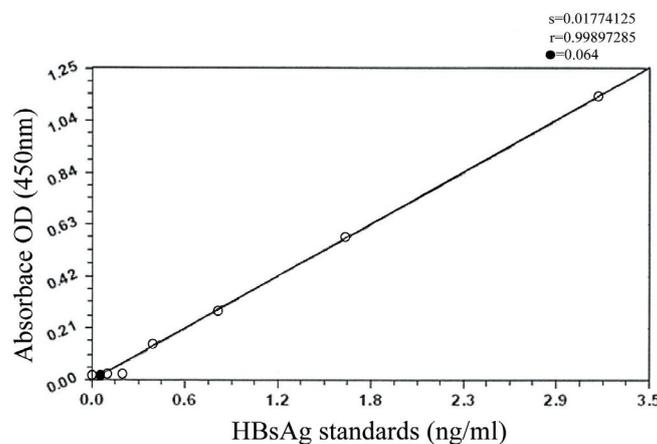


Figure 5. Standard curve for detection of HBsAg (generated by CurveExpert). Standards for HBsAg in the concentration range of 0.0125 - 3.2 ng/ml result in proportional OD₄₅₀ measurements. Regression analysis resulted in the equation $Y = 0.357X - 0.00068$ with a correlation coefficient (R^2) of 0.99897. Based on the standard curve, the average amount of HBsAg detected in a single two-celled embryo is about 0.064 ng/ml.

ELISA

The two-celled embryos exhibiting green fluorescence were selected under a fluorescence microscope and ELISA was performed to establish a standard curve to calculate the HBsAg levels in the individual embryos and to detect the presence of HBcAg in the groups containing one, two, or five embryos, respectively. The average concentration of HBsAg in the individual two-celled embryos was 0.064 ng/ml, according to the standard curve. HBcAg, in contrast, was only detected in the five-embryo experimental group and in the assay positive control, but not in the one- and two-embryo experimental groups or the negative control. The failure to detect HBcAg in the one- and two-embryo groups may be due to a low level of HBcAg in the individual cells of the tested embryos, which when combined are still below the threshold of detection for this specific assay.

DISCUSSION

It has been demonstrated that HBV can infect sperm and the viral DNA can be detected in the sperm nucleus [12,21,22]. In preliminary experiments, the FISH signals for HBV DNA could be detected in the HBV-transfected donor sperm similar to the sperm from the HBV-infected patients [16]. Therefore, donor sperm transfected with the plasmids pIRES2-EGFP-HBs or pIRES2-EGFP-HBc was used instead of patient sperm in the current study, making it possible to distinguish between embryos with and without HBV genes by the expression of green fluorescent protein after fertilization of oocytes by transfected sperm and to determine the antigenicity of the transfected HBV S or C genes.

Replication of Sperm-introduced HBV S and C Genes In Embryonic Cells

The HBV genome is a circular genome of about 3.2 kb in size and consists of DNA that is mostly double-stranded. It has a compact organization, with four open reading frames (S, for the surface, or envelope, gene; C, for the core gene; X, for the X gene; and P, for the polymerase gene) running in one direction and no noncoding regions [18]. HBV is economic with its utilization of its genetic material; this is accomplished by two rare genetic configurations: proteins are encoded from overlapping translation frames, and all regulatory signal sequences reside within protein-encoding sequences. This unusual configuration has led to the

proposal by some scientists that HBV may not use a conservative DNA replication mechanism [18,23]. However, using FISH with the specific HBs- and HBc-DNA probes, respectively, we found that the signals for HBs or HBc DNA could be detected in some of the transfected human sperm nuclei, indicating that the HBV S and C genes integrated into the sperm genome. After fertilization, individual two-celled embryos with green fluorescence had positive signals in both nuclei for HBs- or HBc-DNAs, respectively, suggesting that the sperm-introduced HBV S or C genes were able to replicate concomitantly with the host embryo genome, and the two viral gene copies segregated normally into the daughter cells during the first cleavage. This mode of replication is semiconservative and synchronized with that of the host genome and completely different from the canonical HBV life cycle. The consequences of HBV sequences hidden in the host genome by integration and semiconservative replication reveal a potential new transmission route of HBV, which may induce chromosomal abnormalities, increasing the risk of abortion, stillbirth, or birth defects and may increase the risk of primary hepatocellular carcinoma.

Translation of Sperm-introduced HBV S and C Genes in Embryonic Cells

Using immunofluorescence assays, HBsAg and HBcAg protein were detected within the cytoplasm of embryonic cells. These results suggest that the sperm-introduced HBs and HBc genes are able to express viral proteins in the embryonic cells.

It is now recognized that the host immune responses to viral antigens displayed on infected hepatocytes are the principal determinants of hepatocellular injury [23]. Viral antigens contain highly antigenic epitopes which are responsible for triggering the immune response and cause hepatocyte inflammation. In the current study, it has been confirmed that sperm-introduced HBs and HBc genes were synchronously replicated with the host genome and were competent in protein expression. If HBs and HBc sequences hidden in the host genome can express their antigens, these viral antigens could potentially cause hepatocyte inflammation by triggering an immune response. This situation represents a new mechanism, completely different from the canonical mode of Hepatitis B infection.

Taken together, we have shown, for the first time, direct experimental evidence that sperm-introduced HBV S or C genes are able to replicate synchronously with the host embryo genome in a semiconservative mode of DNA replication, and are able to express viral proteins in embryonic cells. Combined with the findings of our previous studies [16,17,19], we have demonstrated that HBV genes could be transmitted vertically to subsequent generations via the male germ line, and these genes retain their competency to be replicated, transcribed, and translated. However, an HBV gene is not equivalent to an HBV virion, thus further investigation is required to confirm the mechanism for vertical transmission of HBV via the male germ line.

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