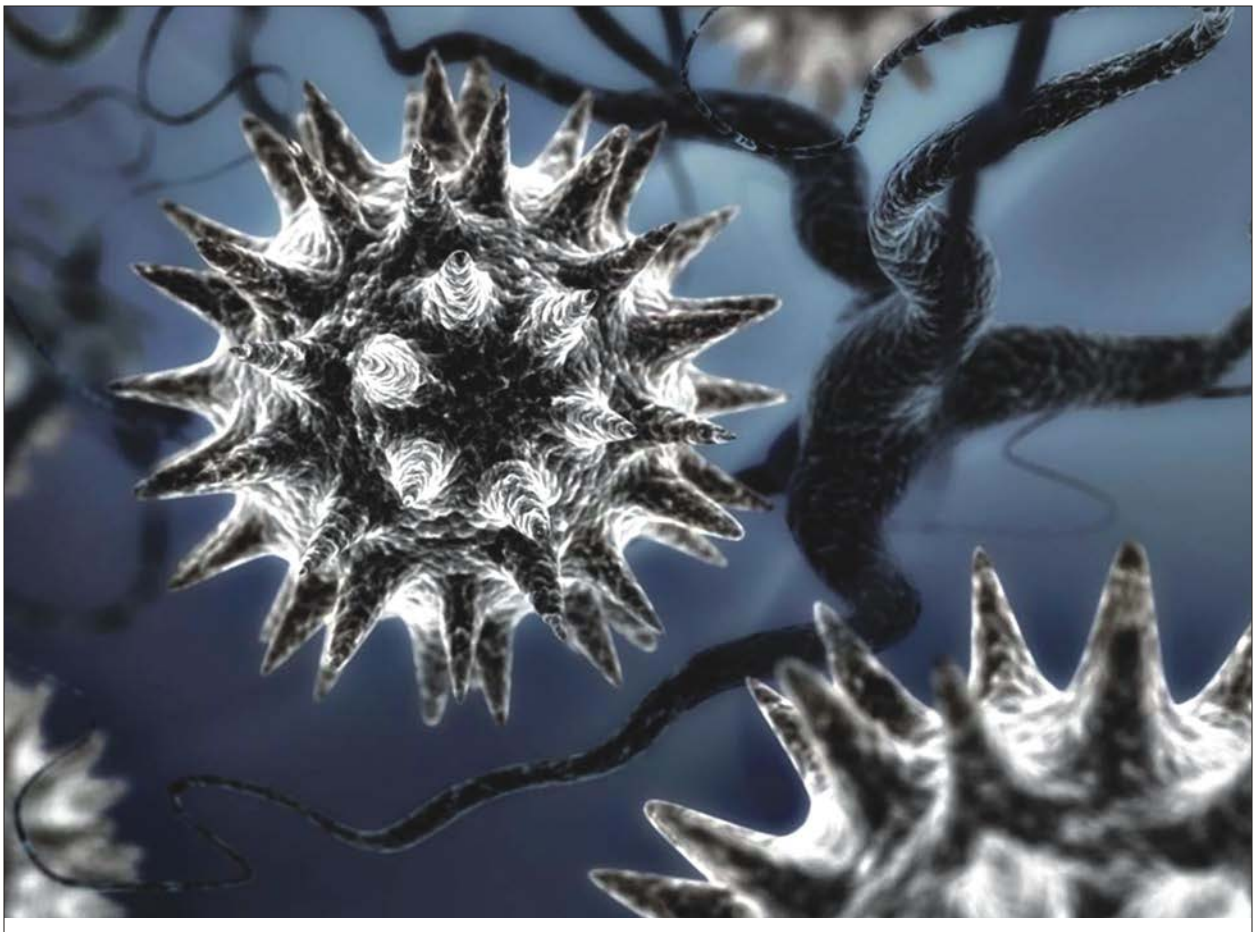

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Detection of Hepatitis B Polymerase Gene in Early Embryonic Cells from Golden Hamster Oocyte and Human Spermatozoa Carrying HBV DNA

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Abstract: The study on vertical transmission of hepatitis B Virus DNA in the human embryo would be an ideal model but such a system presents major logistical, moral and ethical problems. Thus, it is crucial to establish a model system for such study. The present study was designed to amplify HB polymerase gene in one and two cell embryo originated from hamster ova *in vitro* fertilized with human spermatozoa carrying HBV-DNA. Human sperm carrying HBV-DNA was delivered into zona-free hamster oocytes by *in vitro* fertilization assay. HB polymerase gene which is responsible for the reverse transcription of the pregenomic RNA to the double stranded DNA has been detected both in one- and two-cell embryos using PCR. The results reveal that HB polymerase gene is present in one- and two-cell embryo (single embryo) originated from hamster ova *in vitro* fertilized with human spermatozoa carrying HBV DNA sequences. Present results provided the direct evidences that HB-DNA could transmit vertically to next generation via male germ line.

Key words: HB P-gene, embryonic cells, human spermatozoa, hamster ova

INTRODUCTION

The genome of HBV is made of circular DNA, but it is unusual because the DNA is not fully double-stranded. One end of the full length strand is linked to the viral DNA polymerase. The genome is 3020-3320 nucleotides long (for the full length strand) and 1700-2800 nucleotides long (for the short length strand) (Kay and Zoulim, 2007). There are four known genes encoded by the genome called C, X, S and P. The core protein is coded for by C gene (HBcAg) and its start codon is preceded by an upstream in-frame AUG start codon from which the pre-core protein is produced. HBeAg is produced by proteolytic processing of the pre-core protein. HB S gene codes for the surface antigen (HBsAg). The

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HbsAg gene is one long open reading frame but contains three in frame start (ATG) codons that divide the gene into three sections, pre-S1, pre-S2 and S (Beck and Nassal, 2007). The Polymerase open reading frame encodes for a multifunctional enzyme (831 aa; 93 kDa) which is called the polymerase. The polymerase is responsible for the reverse transcription of the pregenomic RNA to the double stranded DNA. Four different domains within the P gene product could be distinguished (Bartenschlager and Schaller, 1988; Chang *et al*., 1990; Radziwill *et al*., 1990). The terminal protein is considered to be the part of the polymerase where the synthesis of the minus strand-DNA is initiated.

In vitro fertilization assay has been widely used by investigators for studying morphological and molecular details of sperm-egg interactions. It well known that the sperm is able to deliver exogenous DNA into oocytes (Brackett *et al*., 1971; Gagne *et al*., 1991; Lavitrano *et al*., 1989; Bachiller *et al*., 1991; Kadze *et al*., 2002). The objective of the present study was to detect HB P gene in early embryonic cells after introducing motile human sperm carrying HBV-DNA into zona-free hamster oocytes via IVF method.

MATERIALS AND METHODS

The experiments of this study as the last part of our research project on HBV vertical transmission during 2006-2008 have been carried out in the Medical College, Shantou University, China. The Institutional Review Board of Shantou University Medical College approved all experiments of this study. Semen sample was given voluntary and taken from a healthy donor (HBV-negative). The IVF assay of zona-free hamster oocytes was performed according to published report (Yanagimachi *et al*., 1976). For a broader discussion of the technique, including the treatment of the semen sample, super ovulation, egg processing, insemination and post-insemination culture, please refer to the following references (Overstreet *et al*., 1980; Kamiguchi and Mikamo, 1986).

Semen Sample Preparation

Semen samples were kept in a CO_2 incubator (37°C, 50 ml L^{-1} CO_2 in air) for 30 min to be liquified. The most highly motile spermatozoa were recovered from the semen with a swim-up method. The sperm suspension thereby obtained was centrifuged at 600 g for 5 min. The pellet was resuspended in fresh Biggers-Whitten-Whittingham (BWW) medium supplemented with 0.3% human serum albumen (HSA, Sigma Chemical Co., St. Louis, MO) and centrifuged again. The washed spermatozoa were suspended in 5 mL of 10 μM Ca^{++} Ionophore (Sigma Chemical Co.) solution for 8 min in the same incubator to facilitate the capacitation. The treated spermatozoa were centrifuged and washed twice with fresh BWW and then suspended in the capacitation medium with 3.5% HSA and incubated for 4 h to allow capacitation of the spermatozoa. Three hours after the beginning of capacitation, human spermatozoa were exposed to the pBR322-HBV plasmid. A total of 100 μL mixture containing 1 μL pBR322-HBV plasmid (1.5 μg mL^{-1}), 6 μL liposome and 93 μL Hepes Buffered Saline (HBS) was incubated at room temperature for 15 min and then added to the capacitation media containing spermatozoa and kept in the incubator for 1 h. After exposing to HBV-DNA, the spermatozoa were washed 5 times in 5 ml fresh BWW via centrifugation at 600 g for 5 min to remove excess HBV-DNA.

Super Ovulation and Oocytes Processing

Mature female golden hamsters (9 weeks old) were housed under standard lighting conditions with free access to water and food. The animals were induced to super-ovulate by intra-peritoneal injection of 30 IU of pregnant mare serum gonadotrophin (PMSG, Ningbo Hormone Product Co., Ltd., Ningbo, China) on day 1 of their oestral cycle and followed

72 h later by administration of 30 IU of human chorionic gonadotrophin (hCG, Ningbo Hormone Product Co., Ltd.). Animals were anaesthetized and killed by cervical dislocation 17 h after hCG injection and then oocytes were collected from the ampullar region of oviducts and freed from cumulus cells by gentle pipetting in 0.1% hyaluronidase (Sigma Chemical Co.). Cumulus-free oocytes were washed twice in fresh BWW medium and treated with 0.1% trypsin (Sigma Chemical Co.) to remove the zona pellucida and then washed twice immediately in fresh BWW.

In vitro Fertilization

As soon as the zona pellucida is removed, the zona-free oocytes inseminated with spermatozoa to prevent their degradation. Insemination was performed with the sperm suspensions at a concentration of about 10^6 mL^{-1}. The oocytes soon began to rotate anti-clockwise due to flagellar movement of the spermatozoa attached to the egg surface. The oocytes were kept in the sperm suspension for only 20 min. The inseminated oocytes were washed twice in fresh BWW in order to remove the excess sperm, then transferred to fresh BWW under mineral oil (Sigma Chemical Co.) and incubated for another 1 h to ensure sperm penetration. After washing twice in fresh Ovum Culture Medium (OCM) (Flow Laboratories, Germany) containing 10% heat-inactivated fetal bovine serum. Each five oocytes were cultured in a droplet (50 µL for each) of OCM under oil in a plastic Petri dish kept in a CO_2 incubator (37°C, 50 ml L^{-1} CO_2 in air) for 24 h. Twenty-four hours after insemination, all embryos were investigated under the microscope (Leica DM IRE2, Leica Microsystem, Wetzlar GmbH, Germany). Each normal one or two- cell embryo was picked up from the culture and washed three times in cold 1XPBS to remove the medium serum. Each embryo from treated group and control group (unfertilized eggs) was transferred individually into 200 µL PCR tube using 0.5 µL 1XPBS, making it possible to store these embryos or to perform Polymerase Chain Reaction (PCR).

PCR Amplifications and Detection of PCR Product

About 4.5 µL of cell lysis buffer was added to each sample, mixed and then incubated at 70°C for 10 min. The cell lysate of each single embryo (one or two cell embryo) was used as a DNA template. The pBR322-HBV DNA and water were used as positive and negative controls, respectively. The primers used in this study were synthesized by Shanghai DNA Biotechnologies Co., Ltd. (Shanghai, China) and designed according to the known HBV genome sequences and the main popular subtype (*adr*) in China. Polymerase gene was amplified using the primers: forward, 5'-TCTTCATCCTGCTGCTATGC-3' and reverse, 5'-CATATCCCATGAAGTTAAGC-3' specific to region 483 bp. Forty five PCR reaction containing 40 pmol of each primer was added to the cell lysate (5 µL) of each sample. The mixture was heated to 94°C for 5 min followed by 31 cycles consisting of 94°C for 30 sec, 57°C for 30 sec and 72°C for 1 min and finally 72°C for 10 min in a Peltier Thermal Cycler (PTC 100). The amplification products were visualized after staining with Ethidium Bromide (EB), after electrophoresis on 1% agarose gel. This experiment was repeated under the same conditions four times.

RESULTS

All PCR products from each single (one or two-cell) embryo were visible with ethidium bromide staining after agarose gel electrophoresis as a single band at the expected sizes 483 bp. No amplification was detected in the negative control (minus template and unfertilized egg) reactions (Fig. 1).

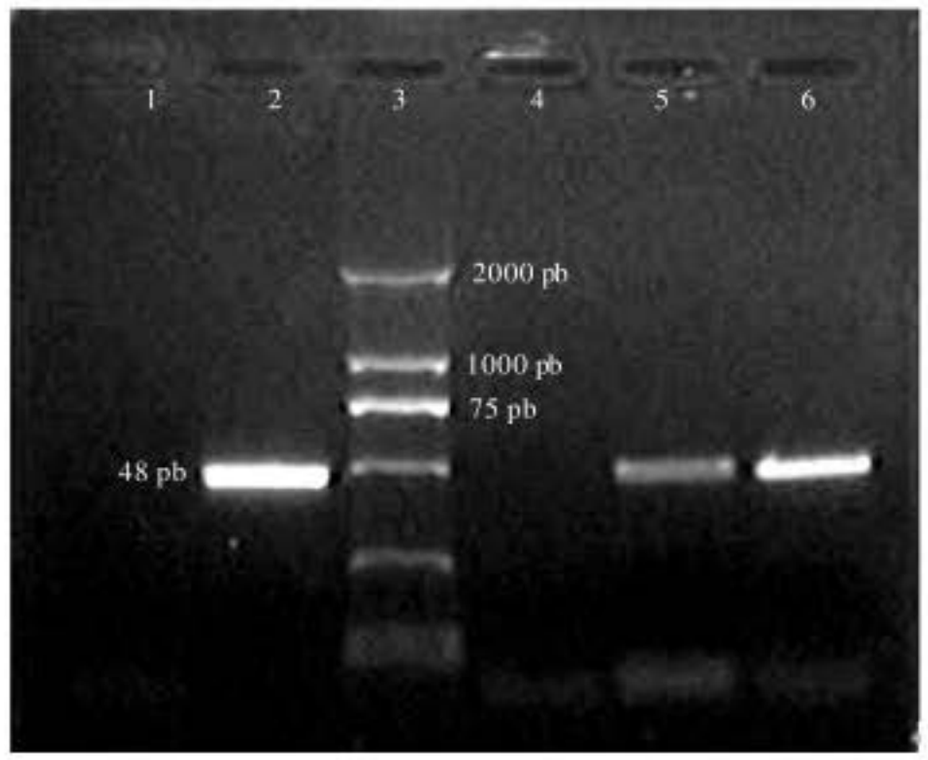

Fig. 1: PCR product (HB polymerase gene 483 bp): Lane 1: Negative control (minus template (double distilled water)), Lane 2: Positive control (HBV-DNA), Lane 3: DNA marker (DL 2,000), Lane 4: Unfertilized egg and Lanes 5 and 6 were from one and two cell embryos, respectively

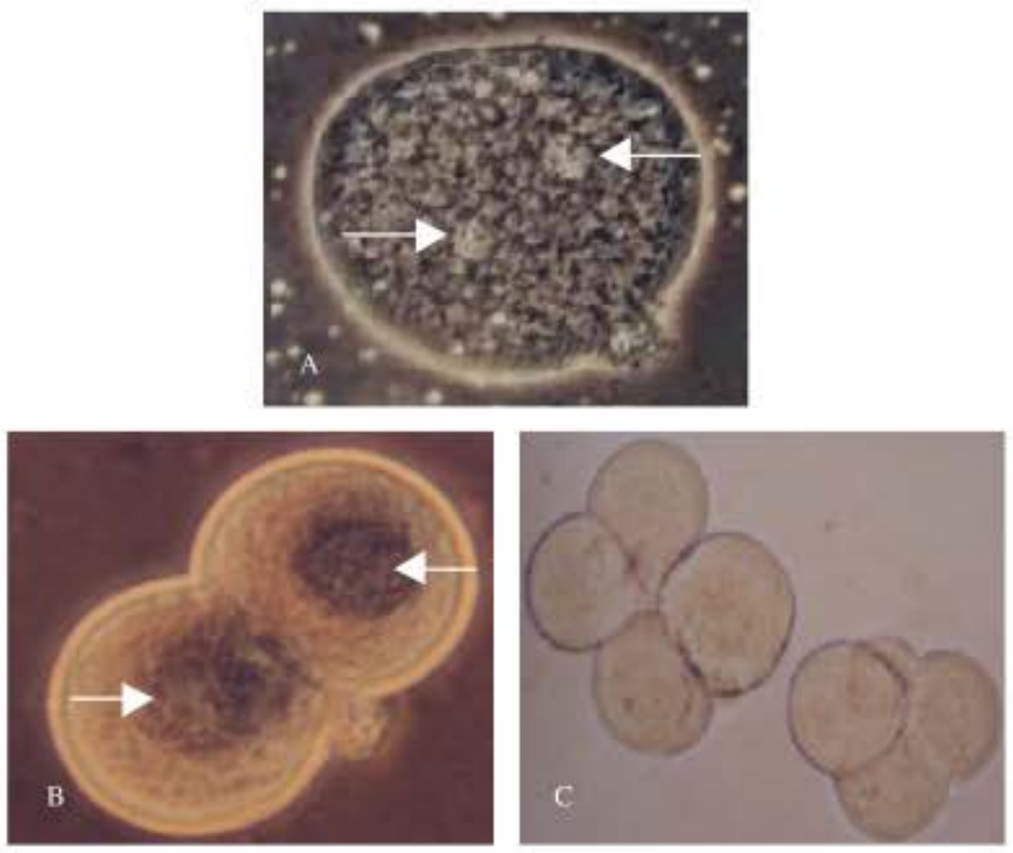

Fig. 2: (A) One-cell embryo showing a male and a female pronuclei, (B) two-cell embryo showing one nucleus in each and (C) four-cell embryo 24 h after insemination (arrows). x400

All amplification products were found to be reproducible when reactions were repeated using the same reaction conditions (Fig. 2A-C). In the present study, we obtained four-cell embryo (Fig. 2C). However, we only used the one- and two-cell embryo to amplify HB polymerase gene, because of the insufficient number for four-cell embryo.

DISCUSSION

The study on vertical transmission of HBV-DNA in the human embryo would be an ideal model but such a system presents major logistical, moral and ethical problems. Thus, it is crucial to establish a model system for such study. To our knowledge, it is the first report to study HB P gene in early embryonic cells. In the present study, interspecific *in vitro* fertilization between human sperm and zona-free hamster ova made it possible. In General, when the zona-free hamster oocyte penetration test is employed, important criteria such as number of eggs penetrated by sperm and number of sperm per penetrated egg is to be assessed. To minimize the chance of polyspermy in our experiments, therefore, the sperm suspension was often diluted more and the oocytes in the fertilization medium were periodically observed under dissecting microscope and those bound by approximately 20-30 spermatozoa were transferred to the sperm-free medium to incubate for approximately 30 min to ensure the higher rate of monospermic penetration.

Single-cell PCRs provide a valuable tool for genetic characterization using a limited amount of starting material. Single embryo at the one or two-cell stage was used for amplification of HB P gene from genomic DNA. The results showed that polymerase gene has been already detected both in one and two cell embryos. The PCR products were obtained as a clear single band at the predicted size (Fig. 1). However, neither specific nor non-specific bands were detected in the negative control reactions (minus template). Present results indicated that the foreign DNA could be integrated into the genome of the progeny. It was impossible that the contamination of washing solutions gave rise to such positive results of PCR in the tested samples because they were washed five times in each experiment.

The present study demonstrated that the HB P gene was integrated into the sperm genome and introduced into the zygote of a normal oocyte via *in vitro* fertilized with spermatozoon. It may well have far reaching implications not only for human health but also for genome reshaping evolutionary processes.

Present results provided the direct evidences that HBV DNA could transmit vertically to next generation via male germ line. The results in this work support the conclusion that human sperm cells can act as vectors for the vertical transmission of HBV genes to the progeny (Ali *et al*., 2005, 2006a, b). This *in vitro* culture system bringing HBV-DNA into zona-free hamster oocytes via human spermatozoa might be used as a model system to study on the mechanism of true vertical transmission of HBV.

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