Detection of Full Length Hb S Gene (1.2 Kb) in One- and Two-cell Embryo Originated from Hamster Oocyte and Human Spermatozoa by Using Nested-PCR

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The polymerase chain reaction (PCR) is a versatile method for rapid amplification of selected DNA segments. The aim of this study was to amplify the full length of Hepatitis B Surface (HBS) gene in one- and two-cell embryo (single embryo) after introducing motile human sperm carrying hepatitis B virus (HBV) DNA into zona-free hamster oocytes via in vitro fertilization (IVF) technique. Human sperm-mediated HBV genome was delivered into zona-free hamster oocytes by IVF. The results revealed that full length HBS could be amplified in one-and two-cell embryo originated from hamster ova in vitro fertilized with human spermatozoa carrying HBV DNA sequences.

Key words: In vitro fertilization, HBS gene, full length, hamster ova, human spermatozoa, nested PCR

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INTRODUCTION

HBV is an enveloped virus and causes acute self-limited and chronic infections in humans (Zheng et al., 2004; Ma et al., 2003). HBV belongs to the hepadnavirus family, containing a small (3.2 kb), circular, double-stranded DNA genome. The minus strand includes at least four open reading frames (ORF), of which S-ORF is divided into pre S1, pre S2 and S gene. The HB S gene contains three regions and encodes for three different glycoproteins, which only differ in the length of their N-terminus. Hepatitis B surface antigen (HBsAg) encoded by the S gene is the major component of hepatitis B vaccine (Kreutz, 2002).

Spermatozoa of a wide variety of species can fuse with zona-free hamster oocytes. Zona-free hamster oocytes were inseminated with spermatozoa of other species such as mouse, guinea pig and human. IVF has been widely used by investigators for studying morphological and molecular details of sperm-egg interactions. It was well known that the ability of sperm to deliver exogenous DNA into oocytes at the time of fertilization has attracted considerable interest and controversy (Brackett et al., 1971; Lavitrano et al., 1989; Bachiiller et al., 1991; Gagne et al., 1991; Fernandez et al., 1999; Kadze et al., 2002).

The study on vertical transmission of HBV gene(s) 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. In the present study, in vitro fertilization between human sperm and zona-free hamster ova made it possible. HBV antigens were detected in human semen and it is now well established that this biological fluid is a vector for the spread of hepatitis B (Darani and Gerber, 1974; Scott et al., 1980; Meheus, 1995; Kleinman et al., 2003). However, few studies have tried to identify the contaminated cells within semen. It has been reported that HBV DNA was integrated into the DNA of spermatozoa in two of three patients with acute hepatitis, suggesting that there may be true transmission of HBV via the germ line (Hadeloual et al., 1985). Our earlier studies have provided the first direct evidence that the HBV gene(s) could be expressed in one and two-cell embryos originated from golden hamster ova in vitro fertilized with human spermatozoa (Ali et al., 2005, 2006). The objective of the present study was to detect the full length of HB S gene in early embryonic cells after introducing motile human sperm carrying HBV DNA into zona-free hamster oocytes via IVF technique.

MATERIALS AND METHODS

Ethical approval: This study was carried out at Research Center for Reproductive Medicine, Shantou University Medical College, People's Republic of China from December 1, 2005 to March 30, 2006. The Institutional Review Board of Shantou University Medical College approved all experiments of this study.

Media: Biggers-Whitten-Whittingham (BWW) medium supplemented with 0.3% human serum albumin (HAS) (Sigma Chemical Co., St. Louis, MO, USA) for human sperm preparation, oocyte collection, insemination and subsequent handling and ovum culture medium (OCM, from Flow Laboratories, Germany) containing 10% heat-inactivated fetal bovine serum for post-insemination culture of ova.

Plasmid: The recombinant plasmid (pBR322-HBV) was kindly supplied by Prof. Hu, YP, Second Military Medical University of China.

Animals: Mature female golden hamsters (8-12 weeks old).

Semen sample: The semen sample semen sample, given voluntarily and taken from a healthy (HBV-negative) donor (a member of the laboratory).

In vitro fertilization method: The IVF assay of zona-free hamster oocytes was performed according to 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; Chaudhuri and Yanoagimachi, 1984; Kamiguchi and Mikano, 1986).

Semen preparations and exposure to recombinant plasmid (pBR322-HBV): Semen samples were kept in a CO₂ incubator (37°C, 50 mL L⁻¹ CO₂ in air) for 30 min in order to be liquefied. The most highly motile spermatozoa were recovered from the semen with a “swim-up” method. The sperm suspension thereby obtained was centrifuged at 600 xg for 5 min. The pellet was resuspended in fresh BWW and centrifuged again. The washed spermatozoa were suspended in 5 mL of 10 μM Ca⁺⁺ Ionophore (Sigma Chemical Co. USA) 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. In brief, 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.

**Oocyte preparation:** Mature female golden hamsters (8-12 weeks old) were housed in groups under standard lighting conditions with free access to water and food. The animals were induced to super-ovulate by intraperitoneal injection of 30 IU of pregnant mare serum gonadotrophin (PMSG, Ningbo Hormone Product Co., Ltd., Ningbo, China) on day 1 of their oestrous cycle, followed 72 h later by administration of 30 IU of human chorionic gonadotrophin (hCG, Ningbo Hormone Product Co., Ltd., Ningbo, China). They 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. USA). Cumulus-free oocytes were washed twice in fresh BWW medium and treated with 0.1% trypsin (Sigma Chemical Co. USA) to remove the zona pellucida and then washed twice immediately in fresh BWW.

**Insemination and post-Insemination culture:** As soon as the zona pellucida is removed, the zona-free oocytes must be inseminated with spermatozoa to prevent their degradation. Insemination was performed with the sperm suspensions at a concentration of about 10⁶ 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. USA) and incubated for another 1 h to ensure sperm penetration. After washing twice in fresh OCM each five oocytes were cultured in a droplet (50 μL for each) of OCM under oil in a plastic Petri dish kept in a CO₂ incubator (37°C, 50 mL L⁻¹ CO₂ in air) for 24 h.

**Embryo investigation:** Twenty-four hours after insemination, all embryos were investigated under the microscope (Leica DMIRE2, Leica Microsystems, Wetzlar GmbH, Germany). A total of 320 golden hamster oocytes were classified into the following groups: normal one-cell embryo having both male and female pronucleus and the second polar body; unfertilized egg containing only female pronucleus; normal two-cell embryo containing two blastomeres with one nucleus each and abnormal two-cell embryo containing two or more nucleus in each blastomere.

**Embryo preparations:** Each normal one or two-cell embryo was picked up from the culture and washed three times in cold 1X phosphate-buffered saline (1XPBS) to remove the medium serum. Each embryo was transferred into 200 μL PCR tube using 0.5 μL 1XPBS, making it possible to store these embryos or to perform nested-PCR.

**Amplification of full length HB S DNA (1.2 Kb):** The primers used in this study (Table 1) were synthesized by Sangon Company, Shanghai, China and designed according to the known HBV genome sequences and the main popular subtype, adr in China. The amplification was carried out using the nested-PCR method according to Ma et al. (2003). Full length HB S DNA was amplified in 50 μL reaction volumes as follows: about 4.5 μL cell lysis buffer were added to each sample, mixed by vortexing 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 and pBR322-HBV DNA and water were used as positive and negative controls, respectively. The 45 μL PCR reaction mix including 40 pmol (each) of the first-round primers (1-2) were added. The mixture were heated to 94°C for 5 min followed by 30 cycles consisting of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min and finally 72°C for 10 min in a Peltier Thermal Cycler (PTC100, USA). Five microliter of the first-round PCR product served as the template for the second-round of PCR amplification which consisting of the same conditions except that the primer pairs and its annealing temperature was raised to 65°C (Table 1). The amplification products were made visible by staining with Ethidium Bromide (EB), after electrophoresis on 1% agarose gel. This experiment was repeated under the same conditions five times.

<table>
<thead>
<tr>
<th>PCR</th>
<th>Primers No.</th>
<th>Primer Sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st-round</td>
<td>1</td>
<td>ACA TCA TCT GTG GAA GGC</td>
</tr>
<tr>
<td>2nd-round</td>
<td>2</td>
<td>TAT CCC ATG AAG TTG AGG</td>
</tr>
<tr>
<td>3rd-round</td>
<td>3</td>
<td>CGG AAT TCA CCA TAT TCT TGG</td>
</tr>
<tr>
<td>4th-round</td>
<td>4</td>
<td>GAA CAA G</td>
</tr>
<tr>
<td>5th-round</td>
<td>5</td>
<td>OCT GCA GGT TTA AAT GTA TAC CCA AAGAC</td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

In general, when the zona-free hamster egg 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. Our preliminary experiments showed that the excessive polyspermy penetration might be resulted from at least one of the following reasons. The final concentration of spermatozoa that actively moved exceeded $10^6$ L$^{-1}$, the large number of spermatozoa attached firmly to the egg surface or the insemination was over a long period (2-3 h). 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 a 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. Moreover, it has been reported that the timing of the events during early development appears to be critical to a successful outcome. In particular, the time of first cleavage division after insemination in vitro is highly correlated with the ability of the embryo to reach the blastocyst stage (Fair et al., 2004).

In the present study, 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 size (1200 bp). No amplification was detected in the negative control reactions (Fig. 1). All amplification products were found to be reproducible when reactions were repeated using the same reaction conditions. 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. This result is in agreement with those of our earlier reports (Ali et al., 2005, 2006) which indicated that parts of HB X, C and S genes from genomic DNA of one or two-cell stage have been successfully amplified. Furthermore, single-cell PCRs provide a valuable tool for genetic characterization using a limited amount of starting material (Gravel et al., 1998). The manipulation and molecular analysis of single cells have many applications in research and medicine. Numerous reports in the literature describe the selective genetic analysis of a very small number of cells, including single cells (Gale et al., 2003). Types of analysis currently performed with single cells include PCR (Hahn et al., 2000); gene amplification (Noo et al., 1995); cDNA libraries (Brady and Iscoe, 1993); DNA damage and repair (Angelis et al., 1999); mRNA differential display (Renner et al., 1998); apoptosis (Piqueras et al., 1996); reverse transcription-PCR (Freeman et al., 1999); gene expression and expression profiling (Dixon et al., 2000). These studies have generated advances in various aspects of cell biology, such as in the analysis of cell-specific gene expression and genetic variations, the characterization of infectious agents for disease mapping and the forensic identification of individuals (Erlich et al., 1991).

CONCLUSIONS

Upon the present results, the HB S gene was integrated into the sperm genome and introduced into the zygote genome of a normal oocyte via in vitro fertilization with spermatozoon. This in vitro culture system bringing HBV DNA into zona-free hamster oocytes via human spermatozoa might be used as a model system for study on the mechanism of true vertical transmission of HBV. The results in this study support the conclusion that human sperm cells can act as vectors for the vertical transmission of HBV genes to the progeny. It may well have far-reaching implications not only for human health but also for genome reshaping evolutionary processes.

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REFERENCES


