The presence and expression of the hepatitis B virus in human oocytes and embryos

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BACKGROUND: The objective of this study was to explore the potential for vertical transmission of hepatitis B virus (HBV) from parents to offspring via human germ cells.

METHODS: For study samples, 250 oocytes from hepatitis B surface antigen (HBsAg) seropositive women and 578 embryos from couples with at least one HBsAg seropositive partner were collected. HBV DNA in the nuclei of oocytes and embryos was detected by fluorescence in situ hybridization; HBsAg expression was analysed using immunofluorescence; and serum HBV DNA levels were measured by real-time PCR. The HBV infection duration of the women and the serum HBsAg status of their mothers were also examined.

RESULTS: HBV DNA was present in 9.6% (24/250) of oocytes and 14.4% (83/578) of embryos. Rates of HBV DNA positive embryos were similar among couples in which the woman, man or both partners were HBsAg seropositive, 13.1% (57/436), 21.3% (16/75) and 14.9% (10/67), respectively. Rates of positivity in oocytes and embryos were significantly higher in a group with high serum levels HBV DNA than in a group with lower serum levels (P = 0.004 and P = 0.002, respectively). Higher rates of oocyte positivity were found for women whose mothers were HBV infected compared with those with uninfected mothers. Expression of HBsAg was observed in 8.7% (2/28) oocytes and 14.1% (10/71) embryos (P = 0.34).

CONCLUSIONS: The presence of HBV DNA in human oocytes or embryos was related to the women’s serum levels of HBV DNA and the infection status of their mothers. The HBV positive embryos were either maternally or paternally dependent. HBV infection may result in vertical transmission to the offspring via germ cells.

Key words: hepatitis B virus / oocyte / embryo / vertical transmission / FISH

Introduction

Hepatitis B is a public health problem throughout the world. According to the most recent World Health Organization (WHO) estimate, more than 350 million people have been infected with hepatitis B virus (HBV) chronically and become carriers of the virus (WHO, 2002). HBV infection is the second most common cause of cancer worldwide (Zuckerman et al., 2007). Hepatitis B is particularly endemic in China (Center for Disease Control and Prevention, 2007). Each year, approximately 300 000 people die from chronic hepatitis B-related liver cirrhosis and hepatocellular carcinoma (Jia and Zhuang, 2007).

The use of the HBV vaccine and the hepatitis B immunoglobulin in the perinatal stage has effectively prevented most perinatal transmissions of HBV. However, 5–10% of infections still cannot be prevented using these methods (Gambarin-Gelwan, 2007; Wang et al., 2008; Willis et al., 2010), and data to explain this phenomenon are lacking. To prevent hepatitis B infection absolutely, we must verify the route of HBV transmission. Some researchers suspect that HBV is possibly transmitted vertically by germ cells.

The possibility of vertical transmission of HBV via human germ cells was first proposed by Hadchouel et al. (1985), who detected the presence of integrated HBV DNA in seminal fluid from a patient with acute hepatitis. Since that time, much attention has been paid to the transmission of HBV by spermatozoa. HBV DNA was even identified in the semen of patients without markers of viral replication in the serum (Davison et al., 1987). In its integrated or free form, HBV DNA was found to be present in spermatozoa (Hadchouel et al., 1985; Naumova et al., 1986; Huang et al., 2002). Using FISH, Huang et al. (2002) found that HBV DNA can integrate into human sperm chromosomes, causing mutagenic effects on chromosomes. HBV S protein (HBs), the main component of the HBV envelope, has adverse effects on human sperm function, and the asialoglycoprotein receptor (ASGP-R) may play a role in the uptake of HBs into the sperm cell (Zhou et al., 2009). It is now well established that

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mature spermatozoa of all species spontaneously take up foreign DNA molecules and deliver them to embryos at fertilization, generating genetically modified progeny (reviewed by Spadafora, 1998; Chan et al., 2000; Smith and Spadafora, 2005; Pitzgoff et al., 2006; Sciamanna et al., 2009). By microinjecting HBV DNA into fertilized mouse oocytes, transgenic mice were produced successfully (Araki et al., 2008). Animal experiments revealed that HBV DNA carried by human spermatozoons could not only enter the zona-free hamster ova through the fertilization process but could also be expressed in early embryonic cells (Ali et al., 2005, 2006; Ahmed et al., 2005, 2006). These studies have provided some evidence that vertical transmission of HBV via spermatozoa containing HBV DNA is feasible.

Studying the presence and expression of HBV DNA in human oocytes and embryos is important for resolving the problem of vertical transmission. At present, only a few studies have indirectly reported that human oocytes could be infected. HBV DNA has been integrated into the chromosomes of hamster oocytes (Huang et al., 2005). HbcAg and HBV DNA may be present in ovaries and ova at different stages of the patient’s infection with HBV (Ye et al., 2006). These studies have hinted at the possibility of HBV vertical transmission by human oocytes.

However, more studies examining the HBV genome in oocytes and embryos are needed to confirm HBV vertical transmission via human germ cells. The main objective of this study was to detect the presence and the expression of HBV in human oocytes and early embryos from patients with HBV infection and to evaluate the influence of the woman’s serum HBV DNA levels, infection duration and mother’s serum HBsAg status on the presence of HBV.

Materials and Methods

Sample collection and processing

Oocytes and preimplantation embryos were collected from the assisted reproductive unit at the Women’s Hospital, School of Medicine, Zhejiang University. Serological screening for hepatitis B was performed as part of the routine workup for all patients undergoing assisted reproductive treatment (ART) in China and included the following tests: hepatitis B surface antigen (HBsAg) and its antibody (anti-HBs); hepatitis B e antigen (HBeAg) and its antibody (anti-HBe); and the antibody for the hepatitis B core antigen (anti-HBc). If the HBsAg was seropositive, preS1 was examined. All the patients were asked about the duration of HBV infection and the serum HBsAg status of their parents. IVF or ICSI was offered according to the semen profile of each male. Sperm were washed using a modified nitroblue tetrazolium chloride/5-bromo-4-chloro 3-indolyl phosphatase solution (ICN) and then viewed with an OLYMPUS AX 70 fluorescent microscope.

Probe labelling and fluorescence in situ hybridization

The recombinant plasmid, pBR322-HBV, containing full-length (3.2 kb) HBV genomic DNA, was amplified according to the routine transformation method. Amplified DNA was labelled by nick translation with ARESTM Alexa Fluor 488 (Invitrogen, England) following the manufacturer’s instructions. Fluorescence in situ hybridization (FISH) was carried out according to (Minné et al., 1998) with some minor modifications. Briefly, slides carrying cells were dehydrated in an ethanol series. The probe solution was applied to the slides under a coverslip, sealed with rubber cement and denatured at 75°C for 5 min. After an overnight incubation at 37°C, the slides were washed in 0.4× SSC/0.3% NP-40 at 73°C for 90 s, followed by 1 min in 2× SSC at room temperature. Finally, the washed slides were mounted with 4, 6-diamidino-2-phenylindole dihydrochloride in an antifade solution. The signal of every cell was observed and interpreted again by two independent observers using an OLYMPUS AX 70 fluorescent microscope.

Dot blot hybridization

The last three washing solutions for the oocyte and embryo preparations were spotted onto positively charged nylon membrane (Osmonics, American) and hybridized with a digoxigenin (DIG)-labelled HBX DNA probe according to the manufacturer’s instructions (Innogenet, China). The plasmid pBR322-HBV DNA and distilled water were used as a positive and negative control, respectively. Oocytes and embryos were not used for FISH unless the last washing solution was free of HBV DNA. After being incubated in prehybridization solution for 1 h, the membrane was incubated for 1 h with gentle shaking in hybridization solution with a DIG-labelled probe. The membrane was washed twice with 2× SSC containing 0.05% SDS at room temperature and then washed twice with 2× SSC containing 0.1% SDS at 50°C. Colour development was achieved using a nitroblue tetrizolium chloride/5-bromo-4-chloro 3-indolyl phosphate solution (Innogenet). The reaction was terminated by washing in distilled water three to five times, followed by photographing the wet filter.

Immunofluorescence

The expression of HBsAg in the oocytes and embryos was detected according to the manufacturer’s instructions (Boster, Wuhan, China). Briefly, oocytes and embryos were washed three times with PBS and fixed for 30 min in 2% paraformaldehyde (Boster) containing 0.5% Triton X-100. After washing with PBS three times, the samples were blocked with a blocking solution containing 4% goat serum for 20 min. Next, the samples were incubated with mouse anti-HBsAg serum (1:200 dilution) for 1 h at room temperature and washed three times with PBS (2 min each). Subsequently, the samples were incubated for 30 min with FITC-conjugated secondary antibody (goat anti-mouse IgG, 1:200 dilution) in the dark and washed three times with PBS (2 min each). Finally, the samples were stained using propidium iodide (PI; Sigma) and then viewed with an OLYMPUS AX 70 fluorescent microscope.
microscope. Positive and negative controls were the same as those described for FISH.

**Quantitative real-time PCR**
The level of HBV-DNA in the serum samples was measured using a commercial diagnostic kit for the quantification of HBV DNA (DA AN GENE Co., Ltd. Guangzhou, China). A quantitative real-time PCR was performed using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) in 96-well microtitre plates. Sample preparation, storage and testing followed the manufacturer’s procedures. The sensitivity of our assays was 100 copies/ml. Each sample run for HBV DNA included a replicate of the negative, low-positive and high-positive controls.

**Statistical analysis**
Statistical analysis was performed using a chi-square test. All statistical analyses were performed in SPSS 16.0. A $P$ value of 0.05 or less was considered statistically significant.

**Results**

**Oocyte and embryo washing solution tested by dot blot hybridization**
To exclude the possibility of HBV DNA transmission from the cell membrane or follicular fluid, dot blot hybridization with DIG-labelled HBX DNA probe was performed. The washing solutions and distilled water (the negative control) were negative. The pBR322-HBV DNA plasmid (the positive control) showed positive signal (data not shown).

**FISH of HBV DNA in oocytes and embryos**
A total of 250 oocytes and 578 embryos comprised: all oocytes from 139 HBsAg seropositive women, 436 embryos from 122 couples in which the woman was HBsAg seropositive, 75 embryos from 27 couples in which the man was HBsAg seropositive, and 67 embryos from 18 couples in which both partners were HBsAg seropositive. One HBV DNA signal (Fig. 1) was detected in 24 oocytes. One to three HBV DNA signals (Fig. 2A–C) were detected in a single nucleus of 83 embryos, but most of the nuclei had only one signal. The positive rate for oocytes was lower than that for embryos, 9.6% (24/250) and 14.4% (83/578), respectively; however, the difference was not statistically significant ($P = 0.06$). The positive rates for embryos were similar for couples in which the woman, man or both partners were HBsAg positive: 13.1% (57/436), 21.3% (16/75) and 14.9% (10/67), respectively ($P = 0.17$; Table I). A HBV DNA signal was detected (Fig. 2D) in the positive controls, whereas the negative controls were negative.

**Serum HBV DNA level and presence of HBV DNA in oocytes and embryos**
According to the serum HBV DNA level, oocytes and embryos from HBsAg seropositive women were divided into two groups: $<10^6$ and $\geq 10^6$ copies/ml. The HBV DNA positive rates for oocytes and embryos were significantly higher in the high HBV DNA group than that in the low HBV DNA group ($P = 0.004$ and $P = 0.002$, respectively), as shown in Table II.

**Infection duration, mother’s serum HBsAg status and HBV DNA in oocytes and embryos**
To examine the relationship between duration of HBV infection of the women and the HBV DNA status of oocytes and embryos, we compared the HBV DNA-positive rates of embryos from the women who were infected HBV pre-puberty with those who were infected post-puberty, and no significant difference was observed ($P = 0.44$). However, the rate of HBV DNA-positive oocytes for pre-puberty infected women was higher than for those infected post-puberty, although the difference was not significant (Table III). Oocytes and embryos from HBsAg seropositive women were classified into positive and negative groups according to their mothers’ serum HBsAg status, respectively. There was a significant increase in rate of HBV DNA-positive oocytes in the women whose mothers were HBsAg seropositive ($P = 0.04$), while the rates of HBV DNA-positive embryos were comparable between the two groups ($P = 0.74$, Table III).

**Insemination method and incidence of positive embryos**
According to the insemination method, the embryos from HBsAg seropositive women, men and both partners were divided into ICSI and IVF groups. Although there were no statistical differences, embryos from HBV-infected men or both partners infected with HBV in the ICSI group had higher rates of HBV positivity than did the embryos of the IVF group, 33.3% (4/12) and 19.0% (12/63), 25.0% (1/4) and 14.4% (9/63) ($P = 0.27$ and $P = 0.48$, respectively, Table IV).

**Immunofluorescence analysis of HBsAg in oocytes and embryos**
To assess the expression of HBV in oocytes and embryos, we examined HBsAg using immunofluorescence. A total of 18 oocytes and 56 embryos were analysed in this study (Fig. 3A and B), including embryos from couples in which the woman, man or both partners were HBsAg positive. Approximately 8.7% (2/28) of oocytes and 14.1% (10/71) of embryos were immunopositive for HBsAg. The rate of HBsAg-positive oocytes was lower than for embryos, but without significant statistical differences.
difference ($P = 0.34$). The HBsAg signal was detected in the positive control, whereas the negative control was negative.

### Discussion

Mature spermatozoa can take up foreign DNA molecules and deliver them to embryos at fertilization (Spadafora, 1998; Chan et al., 2000; Smith and Spadafora, 2005; Pittoggi et al., 2006; Sciamanna et al., 2009). HBV is the prototype member of the hepadnaviridae family, and hepatotropism is a prominent feature of HBV infection. However, it has been reported that HBV infection is not restricted to the liver and has also been observed in semen (Davison et al., 1987; Noppornpanth et al., 2000), spermatozoa (Hadchouel et al., 1985; Naumova et al., 1986; Huang et al., 2002), animal oocytes (Huang et al., 2005; Zhou et al., 2009) and ova at different stages (Ye et al., 2006).

The hypothesis that a new type of vertical transmission occurs via the germ cell requires further research. The study of the presence and expression of HBV DNA in human oocytes and embryos is important to resolve the problem. However, previous research has focused on the influence of the perinatal stage of transmission, either in utero or through exposure to blood at or around birth (Lee et al., 2006), and few researchers have studied the possibility of germ cell transmission. Moreover, due to major moral and ethical problems, it is not possible to do this kind of research on human subjects.

At present, the substantial development of assisted reproductive techniques has enabled us to conduct research on HBV DNA of human oocytes and early embryos. HBV DNA signals were detected in oocytes from HBsAg seropositive women and in embryos from couples in which at least one partner was HBsAg seropositive.

In our centre, sperm used for IVF/ICSI were washed to effectively reduce virus transmission (Sauer et al., 2009; Nicopoullos et al., 2010). In addition, to exclude the possibility of HBV DNA transmission from the cell membrane or follicular fluid, the last three washing solutions were examined by dot blot hybridization. The results suggest that HBV DNA could enter germ cells, and the infected gametes can maintain the ability to fertilize and transfer HBV to the resulting embryos.
In our results, the positive rate for embryos (14.4%) was higher than for oocytes (9.6%). We speculate that fertilization facilitates the detection of HBV DNA. Although the sperm used were washed, small numbers of virus particles under the detection level may still exist (Mencaglia et al., 2005). Following the penetration of sperm, HBV can be introduced into the oocyte and successive divisions of the infected zygote may then result in an infected embryo. When comparing the positive rate of embryos in couples in which the woman, the man and both partners were HBsAg positive, no significant differences were observed. This result suggests that the HBV positive embryos were either maternally or paternally dependent.

A higher positive rate of HBV DNA was found for oocytes and embryos than in previous animal studies (Huang et al., 2005; Chen et al., 2003). One explanation for this is that humans are more susceptible to HBV infection due to species differences. In addition, HBV infection can induce chromosome aberration (Huang et al., 2003), reduce the sperm fertilizing ability (Zhou et al., 2009), increase the incidence of spontaneous abortion and cause preterm labor (Practice Committee American Society for Reproductive Medicine, 2008). The samples used in this study were immature (GV or MI stage) or unfertilized oocytes (MII stage) and poor quality or polyspermic embryos (unsuitable for transfer or cryopreservation), which could provide an explanation for their high infection levels. Whether the presence of HBV DNA has an adverse impact on the fertilization of oocytes and the development of embryos remains unknown, and further studies are needed to investigate this question.

Many studies have shown that the viral load is associated with chronic liver diseases and hepatocellular carcinoma (Chen et al., 2006; Wu et al., 2009; Wong et al., 2010). Massive foreign DNA invasion can activate the reaction of epididymal spermatozoa, but low amounts of DNA cannot (Maione et al., 1997). Serum HBV DNA level is a direct measure of viral presence in oocytes and embryos. The control of serum HBV DNA levels would therefore be expected to lower the risk of viral transmission.

Our results show that the infection duration (pre- and post-puberty) and mothers’ serum HBsAg status of the women was not related to the presence of HBV DNA in embryos, but could be related to the presence of HBV DNA in oocytes. These results suggested that intrauterine exposure of HBV may increase the chance of female germ cell infection, since about 90% of HBV infection is derived from HBsAg- and HBeAg-positive mothers (Chien et al., 2006). Moreover, in early pregnancy, HBV DNA is more likely to enter the immature oocyte before the formation of zona pellucida, which is a protective glycoprotein coat protecting the fragile oocytes (Yanagimachi, 1994).

Information on transmission of HBV in IVF/ICSI is limited. Since a spontaneous pregnancy in patients with HBV infection is accepted, there is no reason for declining infertility treatment. It has been reported that human embryos were accidentally exposed to HBV contaminated in culture medium during IVF, but no infants were infected.
For human immunodeficiency virus (HIV) serodiscordant couples, ICSI may be favoured, because only a single sperm cell rather than millions of sperm are used (Loutradis et al., 2001). However, ICSI procedure bypass the acrosome reaction and penetration through the ZP-two important mechanisms which protect against sperm-borne pathogens and DNA (Kambin and Batzer, 2004), and there is no method to select sperm without virus infection for ICSI. Our results show that for the embryos from HBV-infected men or from two infected partners, the embryos of the ICSI group has higher HBV-positive rate than those of the IVF group, but no significantly difference was detected. The safety of ICSI for HBV infected men remains to be investigated.

Animal research suggests that the exogenous DNA molecules of sperm could propagate as extrachromosomal structures throughout embryonic development (Spadafora, 1998; Pittoggi et al., 2006). More than one HBV DNA signal was detected in the nuclei of some embryos, which suggests that HBV was able to replicate following cell division. It is thought that active HBV would be expressed in the infected cells. We therefore analysed the expression of HBV in oocytes and embryos by immunofluorescence. HBsAg was produced in 8.7% of oocytes and 14.1% of embryos. Because the sample sizes were not large enough, it was difficult to compare the differences in the rates of HBV expression and the serum HBV DNA levels. The HBV DNA in the infected cell possibly exists in a stable form that is not lost during cell division and if the HBV-infected embryo can successfully finish implantation, then the intracellular virus will be continuously amplified in the infected cell. The daughter cells would thus carry the viral DNA forever. Moreover, the exogenous DNA can be integrated into the host genome (Spadafora, 1998; Smith and Spadafora, 2005; Lavitrano et al., 2006), stably transmitted and expressed until an F10 generation (Bagis et al., 2006). These factors may be important for HBV vertical transmission. However, the mechanism through which HBV DNA is propagated and integrated still remains elusive.

Our study design had several disadvantages. Despite being one of the first studies of HBV DNA in human oocytes and embryos, as discussed above, some of our analyses were limited by having small sample numbers. There was no data examining the influence of HBV infection on proper mature oocytes, or on the proper development of embryos and offspring. Moreover, the molecular mechanism governing the virus infection of germ cell is unclear. FISH confirmed the presence of HBV DNA in oocytes and embryos, however, could not distinguish non-covalent linkage from integration site (Arbuckle et al., 2010). Is the integration site of HBV in the nuclei random or specific? Do the infected early embryos successfully finish implantation? If so, are genetic effects of HBV DNA generated? These questions remain to be solved.

In conclusion, this study revealed that HBV DNA could enter the nuclei of human oocytes and embryos. The presence of HBV DNA was related to the serum HBV DNA level, to the serum HBsAg status of the woman’s mother, and possibly to the duration of infection. The presence of HBV DNA in human oocytes and embryos may result in the vertical transmission of HBV. This may be an important mechanism for the transmission of HBV.

**Authors’ roles**

H.X.L. contributed to the conception and design of the research, acquisition of data, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content, and final approval of the version to be published. Z.X.P. contributed to the acquisition of data, analysis and interpretation of data and final approval of the version to be published. Q.Y.L. contributed to the design of the present research, acquisition of the data, analysis and interpretation of data and final approval of the version to be published. W.G.Y. contributed to the acquisition of data, analysis and interpretation of data and final approval of the version to be published. Y.Y.H. contributed to the acquisition of data, analysis and interpretation of data and final approval of the version to be published. Z.Y.M. contributed to the conception and design of the research, and revising it critically for important intellectual content and final approval of the version to be published.

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