Presence of hepatitis B virus in oocytes and embryos: a risk of hepatitis B virus transmission during in vitro fertilization

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Objective: To define the risk of hepatitis B virus (HBV) transmission through oocytes and embryos from chronic HBV carriers.
Design: Laboratory-based study.
Setting: Research laboratory in a university hospital.
Patient(s): Thirty-one couples with hepatitis B surface antigen (HBsAg)-negative women and HBsAg-positive men, 41 couples with HBsAg-positive women and HBsAg-negative men, and 39 seronegative couples.
Intervention(s): None.
Main Outcome Measure(s): Hepatitis B virus DNA and RNA analyses in oocytes and embryos, and the location of virus particles containing HBsAgs.
Result(s): Hepatitis B virus DNA was detected in 3 of 18 male HBsAg-positive/female HBsAg-negative couples (and in 13 of 84 embryos) and 3 of 14 male HBsAg-negative/female HBsAg-positive couples (and in 15 of 71 oocytes and embryos). Hepatitis B virus RNA was detected in 9 of 13 male HBsAg-positive/female HBsAg-negative couples (and in 39 of 52 embryos) and 8 of 17 male HBsAg-negative/female HBsAg-positive couples (and in 30 of 63 oocytes and embryos). The HBsAg, which is present in the nuclei and cytoplasm of oocytes and embryos, was detected in 6 of 10 male HBsAg-negative/female HBsAg-positive couples (and in 13 of 20 oocytes and embryos). Hepatitis B virus DNA, HBV RNA, and HBsAg were not found in 135 oocytes and embryos from 39 seronegative couples.
Conclusion(s): The presence of HBV in oocytes and embryos suggests the possibility of vertical transmission of HBV via the germ line. (Fertil Steril® 2011;95:1667–71. ©2011 by American Society for Reproductive Medicine.)

Key Words: Hepatitis B virus, oocytes, embryos, in vitro fertilization

Hepatitis B virus (HBV) infection is a serious global health problem (1). Two billion people worldwide have evidence of HBV exposure, and an estimated 350 million persons are chronic carriers of HBV (2). In Asia, the chronic HBV carrier rate is estimated to be 8%–10% (2), raising concerns about the safety of assisted reproductive technology (ART) for HBV-infected patients. In our clinical practice, the prevalence of couples with a hepatitis B surface antigen (HBsAg)-positive male partner is 12.61% (436 of 3,457), and couples with an HBsAg-positive female partner represent 7.95% (275 of 3,457) of the total ART patients.

Hepatitis B virus DNA has been found in the ejaculate of HBV-infected patients, either as a free virus in seminal plasma or as an integrated genome in spermatozoa (3–5). When zona-free hamster oocytes were inseminated with human sperm carrying an HBV DNA plasmid using the IVF technique, HBV was able to replicate and was expressed in one- and two-cell-stage embryos (6, 7). In addition, a more recent study has suggested that HBV can transfer from the father to his children (8). However, it is still unknown whether embryos from couples with HBsAg-positive men carry HBV (9).

The possible vertical transmission of HBV via spermatozoa to embryos has been highlighted by the presence of HBV DNA in spermatozoa (3–5). However, recent studies reported that both HBsAg and HBV DNA can be detected in ovarian tissues (10, 11). In addition, Taylor et al. (12) detected HBsAg in follicular fluid. These findings have indicated that the HBV may also be carried by the oocyte.

The risk of transmission of HBV from oocytes and embryos has not been evaluated previously. To evaluate the potential risk of HBV transmission from oocytes and embryos during IVF/intracytoplasmic sperm injection (ICSI), cryopreservation, or gamete donation, we investigated the susceptibility of oocytes and embryos from chronic HBV carriers to HBV during the ART procedure.

MATERIALS AND METHODS

Patients

The inclusion criteria comprised a serologically proven HBV infection (HBsAg positivity) with concomitant tests for antibody to hepatitis B surface antigen (anti-HBs), antibody to hepatitis B core antigen (anti-HBc), hepatitis B e antigen (HBeAg), and antibody to hepatitis B e antigen (anti-HBe) (Abbott Laboratories, North Chicago, IL). We included 41 couples with seropositive women and seronegative men, 31 couples with seronegative women and seropositive men, and 39 seronegative couples. Patients coinfected with

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TABLE 1

Detection of HBV DNA and HBV RNA in oocytes and embryos.

Patients	No. of oocytes and embryos tested		HBV DNA		HBV RNA	
	PCR	RT-PCR	Positive	Negative	Positive	Negative
Couples with HBsAg-positive men (n = 31)	84	52	3 (16.7)	15 (83.3)	9 (69.2)	4 (30.7)
Couples with HBsAg-positive women (n = 31)	71	63	3 (21.4)	11 (78.6)	8 (47.1)	9 (52.9)
Seronegative couples (n = 34)	80	46	0	22 (100)	0	12 (100)

Note: Values in parentheses are percentages.

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hepatitis C virus and human immunodeficiency virus were excluded from this study, as were patients receiving treatment for HBV. The study was approved by the Institutional Review Board of the Tongji Hospital, with informed written consent being obtained from each patient.

Oocytes and Embryos

Ovarian stimulation protocols used for IVF or ICSI were performed using previously established protocols (13). Oocytes were retrieved under ultrasound guidance. After retrieval, oocytes were incubated in 1 mL of IVF medium (Vitrolife, Goteborg, Sweden) and subsequently fertilized by conventional IVF or ICSI and cultured in G-1 medium (Vitrolife) until the day of transfer (day 2 or 3). Serum samples of HBsAg-positive women (n = 31) were collected on the day of oocyte retrieval and stored at −20°C. Immediately after oocyte retrieval, the corresponding follicular fluid of HBsAg-positive women (n = 30) was collected and stored at −70°C. All unfertilized oocytes and nonviable embryos were analyzed immediately.

Twenty oocytes and embryos from seronegative couples artificially contaminated with HBV DNA-positive plasma samples were also tested. Each of the oocytes and embryos was transferred to drops of a plasma sample containing 10^6 copies/mL and incubated at 37°C for 1 h. After incubation, oocytes and embryos were washed six times in cold phosphate-buffered saline (PBS).

Real-Time Polymerase Chain Reaction

The HBV DNA loads in the plasma and follicular fluid were analyzed quantitatively using a real-time polymerase chain reaction (PCR) with an HBV fluorescence quantitative PCR kit (PG Biotech, Shenzhen, China). The assay was standardized using HBV DNA samples of known concentrations from a quality control panel (PG Biotech). The linearity of the assay was stated to range between 500 and 50,000,000 copies/mL. All samples were tested in duplicate.

PCR Amplification

Amplification of HBV DNA from oocytes and embryos was performed according to published guidelines (14). Briefly, oocytes and embryos were washed twice in cold PBS. A pool of oocytes and embryos (3 to 10) of each patient were placed into a microtube with a maximum of 5 μL of media. A 30-μL preamplification solution (50 mM of KCl, 10 mM of Tris-HCl [pH 8.3], and 1.5 mM of $MgCl_2$) was added in each microtube. Samples were centrifuged at 13,000 rpm for 1 minute and heated at 98°C for 15 minutes. Samples were then placed on ice immediately. A final volume of 50 μL was reached by adding 20 μL of PCR amplification solution containing 25 mM of $MgCl_2$, 2 mM of each of four deoxynucleoside triphosphates (dNTPs), 10 pmol/μL of each primer pair, and 0.5 U of *Taq* polymerase (Fermentas, Foster City, CA). The following primers were used: sense primer: 5′GTC TAG ACT CGT GGT GGA CT 3′ and antisense primer: 5′AAC CAC TGT ACA AAT GGC AC 3′. This fragment contained the S region of the HBV genome, and the expected size of the product was 457 bp. For the first amplification, the reaction mixture was denatured at 94°C for 6 minutes and amplified for 40 cycles (94°C for 1 minute; 50°C for 1 minute; 72°C for 1 minute), with an additional 10 minutes at 72°C in the last cycle, using the PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA). The second amplification was carried out in a total volume of 20 μL containing 5 μL of first-round PCR product and 15 μL of PCR master mix: 25 mM of $MgCl_2$, 2 mM of each of four dNTPs, 10 pmol/μL of each primer pair, and 0.5 U of *Taq* polymerase. The samples were amplified under the same conditions as in the first round for 40 cycles. The results were analyzed by running 10 μL of the PCR amplicons on an ethidium bromide–stained agarose gel.

Two negative controls were included. One used unfertilized oocytes and nonviable embryos from seronegative women. The other had all the PCR components, with water substituted for the DNA template. The DNA extracted from HBV-positive HepG 2.2.15 cells served as the positive control. A 294-bp fragment of the human *β-actin* gene amplified from each sample ensured the presence of genomic DNA.

Reverse-Transcriptase PCR

Ribonucleic acid was extracted from oocytes and embryos using the cells-to-cDNA II kit (Ambion, Austin, TX). Briefly, a pool of three to six oocytes and embryos from each patient was washed three times in cold PBS. One hundred microliters of ice-cold cell lysis II buffer was added to the samples, mixed, and incubated at 75°C for 10 minutes. Two microliters of DNase I was added on ice. Samples were then incubated at 37°C for 30 minutes and 75°C for 5 minutes. Ten microliters of each RNA solution was reversely transcribed in a 20-μL reaction mixture containing 4 μL of 10 mM dNTP, 2 μL of 50 μM random decamers, 2 μL of 10× reverse transcriptase (RT) buffer, 1 μL of M-MLV RT (Ambion, Austin, TX), and 1 μL of 10 U/μL RNase inhibitor under the following reaction conditions: 42°C for 30 minutes and 93°C for 10 minutes using the PTC-200 Peltier Thermal Cycler (MJ Research). After complementary DNA synthesis, 5 μL of complementary DNA was amplified

TABLE 2

Serostatus of HBsAg-positive women and HBV-positivity of oocytes and embryos.

Serostatus of HBsAg-positive women	No. of patients	Positive detection of HBV in oocytes and embryos
HBsAg+, HBeAg−, and HBV DNA−	13	1 (7.7)
HBsAg+ and HBeAg+	2	1 (50)
HBsAg+ and HBV DNA+	14	7 (50)
HBsAg+, HBeAg+, and HBV DNA+	2	2 (100)

Note: Vaues in parentheses are percentages. + = positive; − = negative.

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TABLE 3

Detection of HBsAg in oocytes and embryos from HBsAg-positive women.

Patient no.	No. of oocytes and embryos tested	Cell type	Positive
1	2	ZP-free 10-cell embryo, ZP-free 11-cell embryo	2 (100)
2	4	2 ZP-free oocytes, 2 ZP-free 3-cell embryos	4 (100)
3	2	ZP-free oocyte, ZP-free 4-cell embryo	0
4	3	ZP-free 10-cell embryo, 2 ZP-free 6-cell embryos	3 (100)
5	1	ZP-free 3-cell embryo	0
6	1	ZP-free 10-cell embryo	1 (100)
7	3	ZP-free 2-cell embryo, ZP-free 4-cell embryo, ZP-free 12-cell embryo	0
8	2	ZP-free oocyte, ZP-free 6-cell embryo	2 (100)
9	1	ZP-free 9-cell embryo	1 (100)
10	1	ZP-free oocyte	0
Controls	9	4 ZP-free oocytes, 1 ZP-free zygote, 1 ZP-free 2-cell embryo, 1 ZP-free 3-cell embryo, 1 ZP-free 4-cell embryo, 1 ZP-free 8-cell embryo	0

Note: Values in parentheses are percentages.

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as described above. The PCR products were separated on an ethidium bromide–stained agarose gel and visualized by ultraviolet light.

Concurrently, RNA extracted from oocytes and embryos of seronegative women was used as a negative control. Ribonucleic acid extracted from HepG 2.2.15 cells served as the positive control. The *β-actin* gene amplified from each sample served as the endogenous RNA control. To control for the RT and PCR procedures, the minus-RT control and the minus-template PCR control were included.

Immunofluorescence Microscopy

Each of the oocytes and embryos was transferred to drops of Acidified Tyrode's Solution Medium (SAGE In-Vitro Fertilization, Trumbull, CT) until the zona pellucida (ZP) was removed. Samples were fixed in 4% paraformaldehyde (Sigma, St Louis, MO) at 37°C for 30 minutes. After blocking for 30 minutes with 5% bovine serum albumin, samples were incubated overnight at 4°C in the following antibodies: fluorescein isothiocyanate (FITC)-labeled anti-rabbit HBsAg polyclonal antibody (diluted 1:100 in PBS) (AbCam, Cambridge, United Kingdom) or FITC-labeled anti-rabbit IgG polyclonal antibody (diluted 1:100 in PBS) (eBioscience, San Diego, CA), used as the isotype control. The chromatin was stained with 10 μg/mL 6-diamino-2-phenylindole (DAPI; Sigma, St. Louis, MO) for 10 minutes. The samples were then transferred to glass slides and wet mounted. Oocytes and embryos from seronegative women were used as negative controls and were treated as described above. The samples were examined using a confocal laser scanning microscope (Olympus, Center Valley, PA).

Statistical Analysis

The χ^2 or Fisher's exact tests were used for categoric variables where appropriate. Spearman's correlation coefficient was used to estimate the statistical relationship between two quantitative variables. All P values quoted are two-sided, and $P<.05$ was considered statistically significant.

RESULTS

Relationship Between HBV DNA Levels in Plasma and Follicular Fluid of HBsAg-Positive Women

Quantification of HBV DNA was performed in 31 plasma and 30 follicular fluid samples. The follicular fluid of one patient (Patient 18) was not available for testing. No relationship could be established between the HBV DNA load in the plasma and the corresponding follicular fluid ($r = 0.13$, $P=.48$). Thus, the HBV DNA load in the follicular fluid did not reflect the HBV DNA load in the plasma.

HBV DNA and RNA Analyses in Controls and PCR Products

Hepatitis B virus DNA was not detected in 80 oocytes and embryos from 22 seronegative couples. Hepatitis B virus RNA was not detected in 46 oocytes and embryos from 12 seronegative couples (Table 1). The HBV DNA/RNA detection of 20 oocytes and embryos artificially exposed to HBV DNA–positive plasma were positive. The expected 457-bp product was detected in the HBV-positive HepG 2.2.15 cells and the infected oocytes and embryos. In each PCR and RT-PCR assay, the *β-actin* gene from each sample ensured the presence of human DNA/RNA.

HBV DNA and RNA Analyses in Embryos from Couples with HBsAg-Positive Men

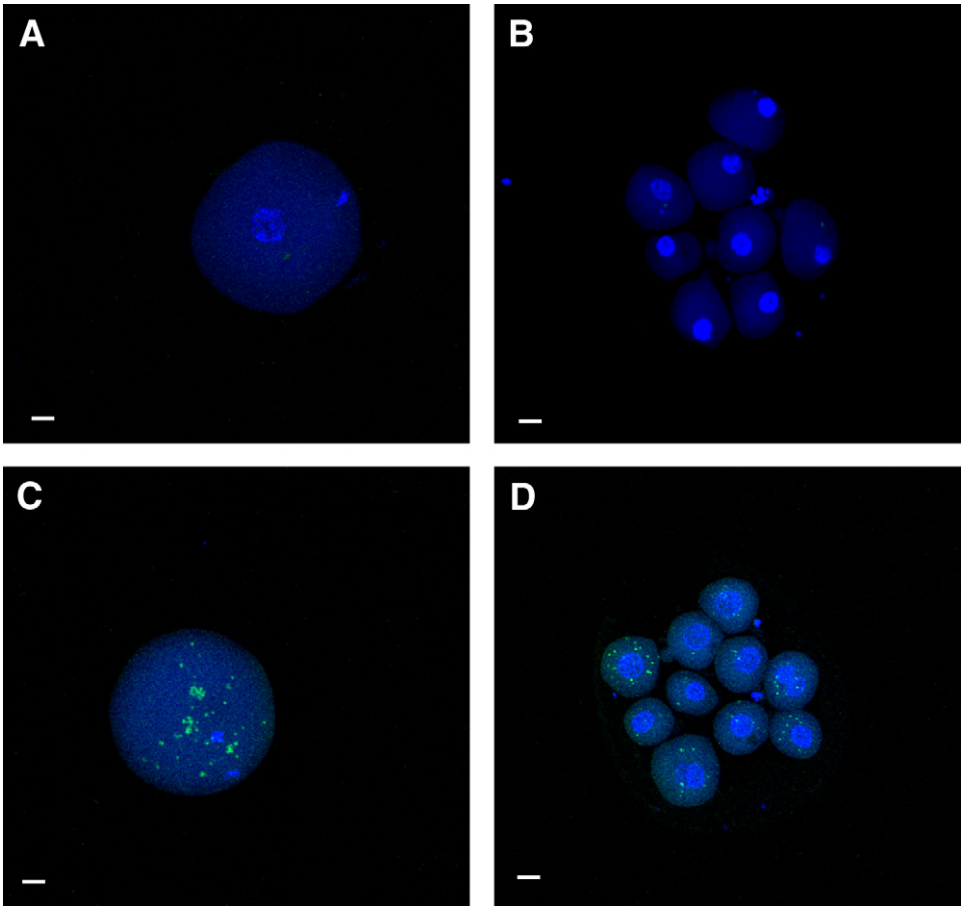
Eighty-four embryos from 18 couples with HBsAg-positive men were subjected to PCR analysis, and 52 embryos from 13 couples with HBsAg-positive men were examined by RT-PCR analysis. Hepatitis B virus DNA was detected in 3 of 18 male HBsAg-positive/female HBsAg-negative couples (and in 13 of 84 embryos), whereas HBV RNA was detected in 9 of 13 male HBsAg-positive/female HBsAg-negative couples (and in 39 of 52 embryos) (Table 1). Of the 5 cases using ICSI and 26 cases using conventional IVF, 40% (2 of 5) of the ICSI cases were positive, compared with 38% (10 of 26) of the conventional IVF cases ($P=1.00$).

HBV DNA and RNA Analyses in Oocytes and Embryos from HBsAg-Positive Women

Seventy-one oocytes and embryos from 14 HBsAg-positive women were subjected to PCR analysis, and 63 oocytes and embryos from 17 HBsAg-positive women were examined by RT-PCR analysis. Hepatitis B virus DNA was detected in 3 of 14 HBsAg-positive women (and in 15 of 71 oocytes and embryos), whereas HBV RNA was detected in 8 of 17 HBsAg-positive women (and in 30 of 63 oocytes and embryos) (Table 1). Of the 8 cases using ICSI and 23 cases using conventional IVF, 13% (1 of 8) of the ICSI cases

FIGURE 1

Confocal microscopic analysis showed the location of HBsAg in oocytes and embryos. Each oocyte and embryo was double-stained for HBsAg (FITC-labeled anti-rabbit HBsAg polyclonal antibody; green) and for chromatin (DAPI; blue). The HBsAg was not detected in the oocyte (**A**) and embryo (**B**) from seronegative couples. The HBsAg was located within the nuclei and/or the cytoplasm of the oocyte (**C**) and embryo (**D**) from HBsAg-positive women. Bars = 10 μm.



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were positive, compared with 43% (10 of 23) of the conventional IVF cases (P=0.20).

For women who were only HBsAg positive, the HBV infection rate of oocytes and embryos was 7.7%. For women who were HBsAg positive and HBeAg or HBV DNA positive, the HBV infection rates of oocytes and embryos ranged from 50% to 100% (Table 2). Among the 20 HBsAg-positive women with HBV-negative oocytes and embryos, HBV DNA loads in the follicular fluid of four women were >500 copies/mL. The amount of HBV DNA in the follicular fluid was <500 copies/mL in 8 of the 11 HBsAg-positive women with HBV-positive oocytes and embryos. The HBV DNA loads in the follicular fluid of the three remaining women were $>10^6$ copies/mL with HBV-positive oocytes and embryos.

Location of HBsAg in Oocytes and Embryos from HBsAg-Positive Women

Twenty oocytes and embryos from 10 HBsAg-positive women, and 9 oocytes and embryos from 5 seronegative couples were examined using direct immunofluorescence. The HBsAg was detected in all ZP-free oocytes and embryos from 6 HBsAg-positive women (60%), whereas the HBsAg was not detected in all ZP-free oocytes and embryos from 4 HBsAg-positive women and 5 HBsAg-negative women (Table 3). The HBsAg was located within the nuclei and/or cytoplasm of the oocytes and embryos (Fig. 1).

DISCUSSION

In our study, HBV DNA was detected in embryos from three couples (16.7%) with HBsAg-positive men, whereas HBV RNA was detected in embryos from nine couples (69.2%) with HBsAg-positive men. This observation is important because it provides direct evidence supporting the hypothesis that the sperm may act as a vector for the vertical transmission of HBV to the embryos, and that the HBV can replicate itself.

Hepatitis B virus DNA was also identified in oocytes and embryos from three HBsAg-positive women (21.4%), whereas HBV RNA was found in oocytes and embryos from eight HBsAg-positive women (47.1%). These results indicate that HBV DNA, which

may be present in oocytes and embryos from HBsAg-positive women, could replicate. Direct immunofluoresence microscopy revealed the presence of HBsAg in the nuclei and the cytoplasm of oocytes and embryos from six HBsAg-positive women, suggesting that virus particles containing HBsAgs may penetrate human oocytes and embryos. Thus, there seems to be a risk of HBV transmission through oocytes and embryos from HBsAg-positive women.

It is of interest that HBV was detected in oocytes and embryos from some but not all chronic HBV carriers. This contradictory result could be explained by the heterogeneous morphology of the ZP. Several studies have described the ZP of a mature human oocyte as a network with multiple pores and hollows (15, 16). In unfertilized human oocytes, superficial pores had a mean diameter of 4 μm, with the diameter decreasing in more inner-lying pores (17). Hepatitis B virus virions are 40–42 nm in diameter (18). Thus, HBV virions may pass through the ZP and reach the cytoplasm. Because human oocytes from the same as well as from different patients have an extremely heterogeneous morphology at the ZP surface (16), we speculate that the HBV may not completely penetrate through the ZP of oocytes and embryos from some women.

We showed that the percentage of HBV-positive embryos was 40% after ICSI and 38% after conventional IVF in male HBV carriers. In the cases in which the women were the chronic HBV carriers, the difference between the percentage of HBV-positive oocytes and embryos in ICSI (13%) and conventional IVF (43%) cases was not statistically significant. Therefore, despite the small size of the study population, there is no reason to advise against an ICSI procedure in chronic carriers of HBV.

In the present study, for women who were only HBsAg positive, the HBV infection rate of oocytes and embryos was 7.7%. For women who were HBsAg positive and HBeAg or HBV DNA positive, the HBV infection rates of oocytes and embryos ranged from 50% to 100%. Because in spontaneous pregnancy the chance of perinatal HBV transmission to the child in the case of a maternal chronic infection is 2%–15% if only HBsAg positivity exists, and this chance rises to 80%–90% if HBeAg positivity or HBV DNA is present (9, 19), it seems that IVF does not increase the risk of vertical HBV transmission. Further, we also found that there was no relationship between the HBV DNA load in follicular fluid and the HBV infection of oocytes and embryos. It was still possible to establish an infection even when the level of HBV DNA in the follicular fluid was low. Therefore, all follicular fluid should be considered as infectious, and consequently the viral risk for retrieved follicles should also be considered.

In conclusion, the presence of HBV in oocytes and embryos suggests the possibility of true vertical transmission of HBV via the germ line. There seems to be a risk of HBV transmission through oocytes and embryos from chronic HBV carriers during the ART procedure.

REFERENCES

1. Dienstag JL. Hepatitis B virus infection. N Engl J Med 2008;359:1486–500.
2. World Health Organization. Hepatitis B. Available at: http://www.who.int/mediacentre. Accessed August 9, 2010.
3. Hadchouel M, Scotto J, Huret JL, Molinie C, Villa E, Degos F, et al. Presence of HBV DNA in spermatozoa: a possible vertical transmission of HBV via the germ line. J Med Virol 1985;16:61–6.
4. Davison F, Alexander GJ, Trowbridge R, Fagan EA, Williams R. Detection of hepatitis B virus DNA in spermatozoa, urine, saliva and leucocytes, of chronic HBsAg carriers. A lack of relationship with serum markers of replication. J Hepatol 1987;4:37–44.
5. Huang JM, Huang TH, Qiu HY, Fang XW, Zhuang TG, Qiu JW. Studies on the integration of hepatitis B virus DNA sequence in human sperm chromosomes. Asian J Androl 2002;4:209–12.
6. Ali BA, Huang TH, Xie QD. Detection and expression of hepatitis B virus X gene in one and two-cell embryos from golden hamster oocytes in vitro fertilized with human spermatozoa carrying HBV DNA. Mol Reprod Dev 2004;70:30–6.
7. Ali BA, Huang TH, Salem HH, Xie QD. Expression of hepatitis B virus genes in early embryonic cells originated from hamster ova and human spermatozoa transfected with the complete viral genome. Asian J Androl 2006;8:273–9.
8. Tajiri H, Tanaka Y, Kagimoto S, Murakami J, Tokuhara D, Mizokami M. Molecular evidence of father-to-child transmission of hepatitis B virus. J Med Virol 2007;79:922–6.
9. Lutgens SP, Nelissen EC, van Loo IH, Koek GH, Derhaag JG, Dunselman GA. To do or not to do: IVF and ICSI in chronic hepatitis B virus carriers. Hum Reprod 2009;24:2676–8.
10. Ye F, Yue Y, Li S, Chen T, Bai G, Liu M, et al. Presence of HBsAg, HBcAg, and HBVDNA in ovary and ovum of the patients with chronic hepatitis B virus infection. Am J Obstet Gynecol 2006;194:387–92.
11. Lou H, Ding W, Dong M, Zhu Y, Zhou C, Wang Z, et al. The presence of hepatitis B surface antigen in the ova of pregnant women and its relationship with intra-uterine infection by hepatitis B virus. J Int Med Res 2010;38:214–9.
12. Taylor PJ, Gill MJ, Mahadevan M, Pattinson HA. Hepatitis B virus in human follicular fluid. Fertil Steril 1987;48:514.
13. Arslan M, Bocca S, Mirkin S, Barroso G, Stadtmauer L, Oehninger S. Controlled ovarian hyperstimulation protocols for in vitro fertilization: two decades of experience after the birth of Elizabeth Carr. Fertil Steril 2005;84:555–69.
14. Witz CA, Duan Y, Burns WN, Atherton SS, Schenken RS. Is there a risk of cytomegalovirus transmission during in vitro fertilization with donated oocytes? Fertil Steril 1999;71:302–7.
15. Vanroose G, Nauwynck H, Soom AV, Ysebaert MT, Charlier G, Oostveldt PV, et al. Structural aspects of the zona pellucida of in vitro-produced bovine embryos: a scanning electron and confocal laser scanning microscopic study. Biol Reprod 2000;62:463–9.
16. Magerkurth C, Töpfer-Petersen E, Schwartz P, Michelmann HW. Scanning electron microscopy analysis of the human zona pellucida: influence of maturity and fertilization on morphology and sperm binding pattern. Hum Reprod 1999;14:1057–66.
17. Nikas G, Paraschos T, Psychoyos A, Handyside AH. The zona reaction in human oocytes as seen with scanning electron microscopy. Hum Reprod 1994;9:2135–8.
18. Ganem D, Prince AM. Hepatitis B virus infection-natural history and clinical consequences. N Engl J Med 2004;350:1118–29.
19. Steyaert SR, Leroux-Roels GG, Dhont M. Infections in IVF: review and guidelines. Hum Reprod Update 2000;6:432–41.