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Efficacy and Genotoxic Effect of Entecavir: the Application in Preventing Father-to-Infant Vertical Transmission of Hepatitis B Virus

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Abstract

This study aimed to assess the clinical efficacy and genotoxic effect of entecavir in preventing father-to-infant vertical transmission of the hepatitis B virus (HBV). A total of 103 couples were included in the observational group. All the fathers were positive for HBV markers including HBsAg, HBeAg, anti-HBc, and HBV DNA, In the control group, 106 couples were recruited with fathers who were positive for the aforementioned HBV markers and mothers who were HBsAg-negative and anti-HBs-positive. Meanwhile, genotoxic effect of entecavir was explored. In the observational group, all the neonates were HBsAg- and HBV DNA- negative, but anti-HBs-positive, which suggests that father-to-fetus HBV vertical transmission was completely blocked. In the control group, 84 out of 106 (79.2%) neonates were anti-HBs-positive, which were significantly different from the observational group. In the DT40 genotoxic research , The data strongly suggest that entecavir is genotoxic ,while in our clinical study ,There were no significant differences between the two groups in terms of gestation week, birth weight, birth length, Apgar scores at 1 min and 8 min, pathologic jaundice, other internal and surgical diseases, and delivery modes. Furthermore, there were no fatal malformations and dead fetuses in the two groups. Our clinical study suggests that antiviral therapy in HBV DNA-positive fathers before conception was able to reduce the HBV viral loads, and to a large extent, block father-to-fetus vertical transmission. The use of entecavir in such fathers makes it safe for conception(the registration number:ChiCTR-OOC-16009151).

Introduction

In 1985, Hadchonel et al [1] found that the DNA of the hepatitis B virus (HBV) was able to integrate into the sperm genome of three acute HBV patients through molecular hybridization. This was the first time that molecular evidence of father-to-infant transmission of HBV was discovered. The development of genetically modified technology has allowed scholars [2,3] to test the hypothesis of HBV transmission via the male germ line. Hybrid signals were detected by viral probes in human sperm chromosomes after fertilization of a zona-free hamster oocyte by sperm from HBV-patients. These results demonstrate that human sperm containing integrated HBV DNA were not be subjected to selective elimination and successfully completed the fertilization process. It has been proved that sperm actively integrates the HBV DNA, with their activity and capability of fertilizing an egg for further development remaining intact [1]. In 2006, Ali et al observed sperm-mediated HBV gene replication in early embryonic cells, providing direct evidence for father-to-infant transmission of HBV [5]. Tajiri H et al also found molecular evidence for HBV transmission from father to infants [6]. Meanwhile, HBV markers were detected in multiple organs in a dead fetus with a HBV-carrier father (mother negative for HBV), which preliminarily identified the potential for prenatal HBV transmission from the father to the infant [7] Modes of father-to-infant HBV transmission include horizontal and vertical transmission. Horizontal transmission refers to HBV transmission from fathers to neonates via daily contact, which could be prevented by a combination injection of hepatitis B immunoglobulin (HBIG) and hepatitis B vaccine (HBVac). Vertical transmission of HBV primarily depends on germ cells. Fathers who are HBsAg-, HBeAg-, and anti-HBc- positive are prone to transmit HBV to their children. Currently, HBV infection of children from their carrier fathers has become the second most important route of transmission of HBV [8]. Vertical transmission of HBV could be via mother-to-infant or father-to-infant routes [9] To date, some progress has been made on the HBV mother-to-infant transmission route and prophylaxis. Effective and safe anti-HBV drugs are able to block mother-to-infant HBV transmission via the inhibition of viral replication in pregnant mothers with high HBV loads. It has been reported that lamivudine can block HBV intrauterine infection to a certain extent [10].

Approaches for blocking father-to-fetus vertical transmission of HBV have drawn much attention from researchers worldwide. The present study aimed to investigate the clinical efficacy and genotoxic effect of entecavir, a guanine analogue, for blocking HBV transmission from a father to a fetus.

Materials and Methods

Clinical practice

Case selection

This study included an observational and control group. The ratio of observation group and control group needs to try to control in 1:1.

In the observational group, a total of 114 fathers were randomly selected according to seeing a doctor order from March 2010 to June 2013 in Qinhuangdao Third Hospital, but there were 8 couples to withdraw because informed consent were not voluntarily signed, the other 3 couples (3infants) were relocation to another city.103 couples had good compliance and accepted follow-up, All children have been completed follow-up in July 2015. The status of HBV markers in the fathers were HBsAg-positive, HBeAg-positive, anti-HBc-positive, and HBV DNA-positive. Seventy-nine fathers with hepatic dysfunction accounted for 76.7% (79/103) of the observational-group participants. Moreover, fathers who were positive for HBsAg, anti-HBe, anti-HBc or HBsAg, anti-HBc, and HBV DNA were excluded from this study. The mothers selected had normal liver function. Sixty-three mothers were positive for either anti-HBs or anti-HBs, anti-HBc or anti-HBs, anti-HBe and anti-HBc. Forty mothers were negative for anti-HBs, accounting for 38.8% (40/103) of mothers in the observational group. The inclusion criteria for all couples in the observational group were fathers who were negative for HBV DNA and had normal liver function before conception, and mothers who were positive for anti-HBs.

In the control group, a total of 116 cases were randomly selected from couples who had undergone prenatal tests according to seeing a doctor order in the Women and Children's Hospital of Qinhuangdao from March 2010 to June 2013. but there were 10 couples to withdraw because informed consent were not voluntarily signed, the other 106 cases had good compliance and accepted follow-up, All children have

been completed follow-up in July 2015. Fathers had normal liver function and were positive for HBsAg, HBeAg, anti-HBc, and HBV DNA. There were 2 fathers with HBV DNA loads of ≦ 10e6IU/ml and 104 cases with > 10e6 IU/ml. There were 106 pregnant mothers with normal liver function who tested negative for HBsAg, positive for anti-HBs or positive for anti-HBs and anti-HBc or positive for anti-HBs, anti-HBe and anti-HBc. Other mothers who were negative for HBV markers and negative for anti-HBs and positive for anti-HBc were excluded from this study.

Eligibility criteria for inclusion in this study were couples who tested negative for serum hepatitis A, C, D, and E viruses, and HIV, without any alcoholic liver disease, drug-induced liver injury, and autoimmune liver disease. They were also required to have normal renal function. Informed consent forms were signed by all couples. All fathers had a medical history of 2 to 25 years. Venous blood from the neonates was harvested. This study was approved by the Ethical Committee of Qinhuangdao Third Hospital (**Figure 1**).

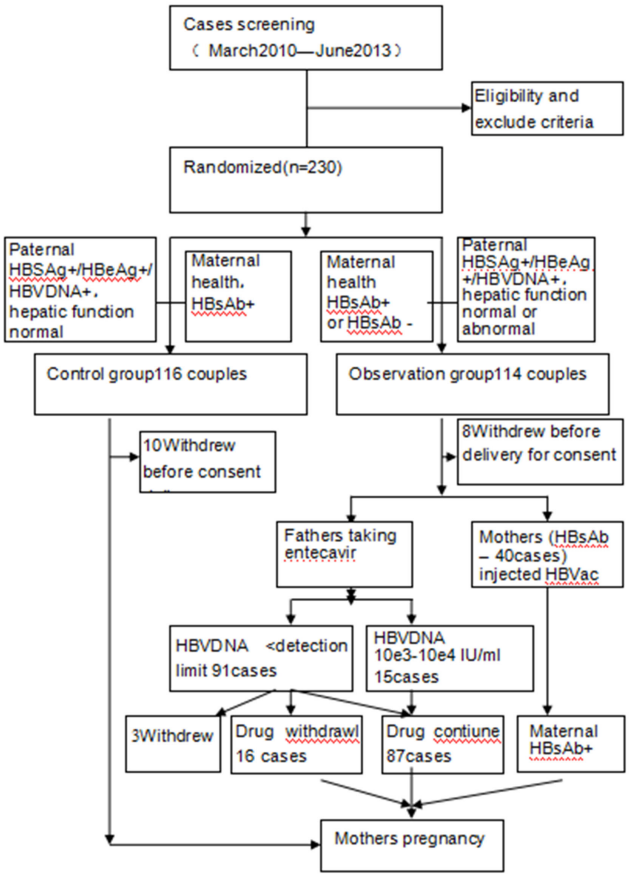

Figure 1. A flow chart for parents enrollment and pregnancy.

Observational index

In the observational group, the liver function, HBV markers, and HBV DNA of fathers were measured before anti-viral therapy and after treatment at 1, 3, 6, 9, and 12 months. Maternal liver function and HBV markers were also examined. Among these mothers, 40 tested negative for anti-HBs and underwent anti-HBs tests at 1 and 7 months.

In the control group, the liver function and HBV markers were examined in all the mothers and fathers. In addition, the HBV DNA levels of the fathers were determined.

The levels of HBV markers and HBV DNA were measured in the venous blood of the neonates immediately after birth.

Methods

The same samples in the observation group, control group, and neonates were used. HBV markers and HBV DNA were detected by an electrical chemiluminescence immunoassay (Roche) and a real time polymerase chain reaction (PCR, Shengxiang Biotech, China), respectively [11].

Treatment

In the observational group, 40 mothers were anti-HBs-negative, and vaccinated with the HBVac (0, 1, 6, recombinant yeast 20, or 40 μg). The other 63 cases were anti-HBs-positive and were not subjected to HBVac injection. All the 103 mothers were positive for anti-HBs before pregnancy. The fathers that were HBV DNA-positive were treated with entecavir at a dose of 0.5 mg/day before conception. Drugs based on entecavir instruction（entecavir went on the market in China in 2005）. Entecavir in instruction for a summary description of the reproductive toxicity and pregnancy drug, no found male and female rats fertility affected, When dose to 35 times human doses or more, Discovered the rodentia animal and the dog to appear the vas deferens degeneration, In monkey experiment, no found testicles changed.

In the control group, 106 mothers were anti-HBs-positive and were not subjected to any treatment during gestation. None of the HBV carrier fathers underwent any treatment before conception.

Genotoxic effect of entecavir

Chemicals

Entecavir was obtained from Sigma-Aldrich (St. Louis, MO, USA,lot no. : 084M4716V). CPT (Camptothecin) was purchased from Shanghai standard

Biotech Co., Ltd（lot no.：83/091221）. Stock solution of entecavir (100 μM) and CPT (100 μM) were prepared in dimethyl sulfoxide (DMSO) and stored at -20°C in aliquots until use.（lot no.：2094C359）, MTT(Amresco,USA,lot no.：20130108415）,RPMI-1640(Gibco,lot no.：8115392）, β-mercaptoethanol(Gibco,lot no.：1628448）, L-Glutamine 200Mm(100×)(Gibco,lot no.：1606139）, Penicillin-Streptomycin Solution(Gibco,lot no.：SK15119）colcemid (Gibco,USA,lot no.：1559025）, Giemsa solution(Nanjing Jiancheng technology Co., Ltd ,lot no.：20151110）, KCl(Chengdu kelong chemical reagent factory,lot no.：20120301）, NP-40(Beyotime,item no.：ST366）,Albumin BovineV(Solarbio,item no.：A8020）,Anti-γH2AX(Ser139) mouse monoclonal antibody(Millipore,USA,lot no.：2138016）,Alexa Fluor488 -conjugatedanti-mouse antibody(Beyotime,Wuhan, China,item no.：A0216）, PBS(item no.：AR0030）,4%Paraformaldehyde（item no.：AR1069）

Experimental cell source

All used DT40(the chicken B cell line) were provided by professor Shunichi Takeda in Kyoto University

Method

MTT assay

The cytotoxicity of entecavir or CPT on DT40 cell lines was determined by the MTT assay [12,13].

Chromosomal aberrations analysis

Karyotype analysis was done as previously described [14].

Immunofluorescent

Experimental condition for immunofluorescent analysis is described previously [15].

Statistical analysis

Statistical analysis was performed using SPSS for Windows (Version 16.0, SPSS Inc., Chicago, USA) Measurement data and categorical data were analyzed with t tests and X^2 tests. Analysis of covariance(ANCOVA) was used to test for differences in the linear dose-response curves between wild-type and a series of mutant cells. A value of $P < 0.05$ was considered significant.

Results

Prenatal Situations of fathers and mothers in the observational and control groups (Table1).

Neonates in the observational group and control group (Table2)

In the observational group, 103 fathers were treated with entecavir. Among these couples, 87 women become pregnant while their spouses were undergoing antiviral treatment. After treatment with entecavir, 16 fathers became HBVDNA-negative and were withdrawn from the antiviral drugs between three to six months. During this time, their wives became pregnant (**Figure 1**).

HBV infection status in neonates

In the observational group, 87 females were pregnant during the period in which their spouses were taking medication. Among them, 72 mothers ' conception came after their spouses were tested negative for HBV DNA. There were no neonates positive for HBsAg and HBV DNA. The other 15 fathers with normal liver function were still positive for HBV DNA (1.0 × 10e3 - 9.1 × 10e4 IU/ml). Although their fathers were HBV DNA-positive, the neonates were HBsAg- and HBV DNA-negative.

In addition, 16 fathers become HBV DNA-negative after treatment with entecavir and were withdrawn from the antiviral drugs between three to six months. Their wives became pregnant, and the neonates all tested negative for HBsAg and HBV DNA. In the control group, 84 out of 106 (79.2%) neonates were anti-HBs-positive, 13 (12.3%) were HBV DNA-positive, and 9 (8.49%) were HBsAg-positive. Six neonates (5.66%) were anti-HBs- and HBV DNA-negative (**Table3**).

DT40 genotoxic effect

Mutant cells defective in DNA repair pathways were sensitive to entecavir, Entecavir induced the accumulation of γ-H2AX in nuclei of DT40 cells, DNA repair-deficient cells showed a marked increase in entecavir-induced chromosome breaks [16].

Discussion

There is a high prevalence of HBV infection in China. The use of the HBVac is helping to bring HBV transmission under control. A national epidemiological survey on HBV in 2006 indicated that 7.18% of people aged 1 to 59 years old carried HBsAg, and that only 0.96% of children under 5 years old were HBsAg-positive [17,18]. Meanwhile, epidemiological survey data revealed that HBV infection has an obvious familial aggregation [19,20]. A study by Wang et al confirmed the genotyping consistency of HBV between

the father (mother without HBV infection) and his child. I ›mology of HBV largely

chromosomes at multiple sites in a non-specific way, which increases the instability of the chromosomes. In

Table 1. The situation prenatal of the parents in the two groups.

	Cases (n)	Antiviral treatment before conception(n) (fathers)	Quantitative anti-HBs prenata l(IU/L) (mothers)
Case	103	103	457±34.6
Control	106	0	491±37.5
x^2(t)-value		x^2=209.00	t=-1.154
p values		0	0.313

Note: Anti-HBs, antibody against hepatitis B surface antigen.

Table 2. General situation of the neonates at birth in the two groups.

Observed indicators	Pregnancy week	Weight (kg)	Height (cm)	Gender (M / F)	One min Apgar score	Eight min Apgar score	Jaundice (n)	Other internal and surgical diseases	Delivery mode (caesarean/head)
Case group (n=103)	39.11±1.30	3.42±0.32	48.73±1.56	57/46	9.83±0.52	9.87±0.54	3	0	45/58
Ctrol group (n=106)	39.31±1.31	3.35±0.33	49.42±1.53	55/ 51	9.80±0.51	9.86±0.57	4	0	45/ 61
x^2 (t)	t=-0.188	t=0.264	t=-0.547	x^2=0.250	t=0.071	t=0.022	x^2=0.120		x^2=0.033
p-values	0.860	0.805	0.613	0.617	0.947	0.983	0.729		0.857

Note: M, male; F, female.

Table 3. HBVM status of the neonates at birth in the two groups.

	Cases (n)	anti-HBs (+)	HBsAg (+)	HBVDNA (+)
Case	103	103	0	0
Control	106	84	9	13
x^2		23.892	9.139	13.470
P-value		0.000	0.003	0.000

Note: HBVM, hepatitis B virus marker; anti-HBs, antibody against hepatitis B surface antigen; HBsAg, hepatitis B surface antigen.

2004, Englert et al [24] reported that gradient centrifugation in assisted reproduction technology (ART) reduced HBV viral loads and prevented embryo contamination. Although washed sperm could decrease HBV transmission, the clinical application of ART is limited when

precluded the possibility of mother-to-fetus transmission, and indicated that HBV in infants was transmitted from the father. This study provides molecular evidence for the transmission of HBV from a father to his child [21]. In 2014, Zhang et al [22] explored the effects of paternal semen HBV DNA on the vertical transmission of HBV and elucidated a dose-response relationship of HBV DNA levels between the paternal serum and semen. Their findings revealed that paternal serum that was HBV DNA- and HBeAg-positive, and semen that was HBV DNA positive were all risk factors for father-to-fetus HBV transmission. In addition, paternal serum HBV DNA loads of > 10e5 IU/ml and semen HBV DNA loads of > 10e3 IU/ml increased the positive rate of father-to-fetus vertical transmission. Huang et al [23] found that HBV infection was able to induce the mutation of sperm chromosomes. HBV DNA integrates with sperm

blocking father-to-infant vertical transmission of HBV. Therefore, there is an urgent need to find convenient and effective ways to prevent father-to-fetus HBV transmission.

Characteristics of neonates

Nucleoside analogues can be used against HBV replication. They act as chain terminators and stop viral DNA polymerase owing to their similarity to nucleotides, which allows them to be incorporated into growing DNA strands. In recent years, antiviral drugs that are nucleoside analogues have been widely used for the treatment of chronic HBV. Due to the long course of treatment, suspension of the medication is only possible in a quarter of HBV patients, while the majority has to take the medication for a long time. During this treatment period, many patients will be at a childbearing age. Interferon treatment produces a

negative effect on male sperm. It has been reported that PEGylated interferon alpha and ribavirin decrease sperm counts, and lead to sperm and sperm chromosome abnormalities [25], which can be recovered eight months after drug withdrawal. Thus, interferon is not appropriate for males of childbearing age due to its negative effects on sperm. To date, there is no evidence suggesting that therapeutic doses of nucleoside analogues induce sperm abnormalities. Entecavir, a carbocyclic 2'-deoxyguanosine analog, was widely used for HBV clinical therapies by inhibiting the HBV polymerase, competing with dGTP, The guidelines for reproductive toxicity and the use in pregnancy of entecavir state that there is no evidence showing the reproductive toxicity of entecavir in male and female rats. The toxicological study reveals that entecavir treatment at a dose of 35 times or more of that used in humans results in spermaduct degeneration in rodents and dogs. Experimentation in monkeys demonstrated no testicular changes induced by entecavir. Taken together, these results demonstrate the safety of entecavir in humans, except in the cases of drug overdose-induced toxicity and potential carcinogenicity. but in the DT40 genotoxic research: we used the concentration of entecavir from 4 to 64 nM, which was based upon the maximal clinical exposure concentration.

30nM [26,27], to analyze the sensitivity of a panel of DNA repair deficient DT40 cells to entecavir,The data strongly suggest that entecavir is genotoxic [16] ,while in our clinical study, there were no significant differences in the two groups in terms of gestation week, birth weight, birth length, Apgar scores at 1 min and 8 min, pathologic jaundice, other internal and surgical diseases, and delivery modes. Additionally, there were no fatal malformations and dead fetuses in the two groups. The aforementioned indices were not influenced by the vertical transmission of HBV from fathers for HBsAg-positive to fetuses [28]. Other investigations found that HBV-carriers exhibit a higher risk of sperm chromosomal aberration, sterility, miscarriage, stillbirth, perinatal infant mortality, and fetal malformation compared to healthy controls [29-31]. The use of entecavir in such carriers makes it safe for conception. When assessing if the benefits outweigh the risks, men intending to conceive could continue with the antiviral therapy. As the Chinese guidelines for chronic HBV state, nucleoside drugs have not been reported to have negative effects on the sperm of males infected with HBV [32]. Thus, pregnancy could be considered on the premise of communicating all the necessary information to patients. From the perspective of eugenics, we will continue to evaluate the safety of entecavir with a larger sample size and long-term follow-up.

Father-to-infant HBV vertical transmission

The placenta begins to actively transmit IgG to the fetus after 20 weeks of gestation. Ayoda et al [33] showed that 59% of neonates whose mothers were vaccinated with the HBVac were HBsAb-positive and HBV-negative; therefore, suggesting the acquisition of immunity against HBV in utero.

Previous studies showed that neonates whose fathers were positive for HBsAg had higher HBV infection rates [34,35]. However, these studies did not reach a consistent conclusion in terms of the HBV-transmission rates [36,37] Such inconsistency could possibly be due to the inconsistent diagnostic criteria and discrepancies in the studies objects. In our study, all the neonates in the observational group were HBsAg- and HBV DNA-negative, but anti-HBs-positive, suggesting that father-to-fetus HBV vertical transmission was completely blocked. In the control group, 84 out of 106 (79.2%) neonates were anti-HBs-positive, 13 (12.3%) were HBV DNA-positive, and 9 (8.49%) were HBsAg-positive, which were significantly different from the observational group. These data suggest that antiviral therapy in HBV DNA-positive fathers prior to conception could reduce the HBV viral loads largely block father-to-fetus vertical transmission. In the future, we will continue to investigate the stratification of paternal HBV DNA and maternal anti-HBs status with a larger sample size.

Conclusions

Our study suggests that antiviral therapy in HBV DNA-positive fathers before conception was able to reduce the HBV viral loads, and to a large extent, block father-to-fetus vertical transmission. The use of entecavir in such fathers makes it safe for conception. After assessing whether the benefits outweigh the risks, it was determined that it may be beneficial for men intending to conceive to continue with the antiviral therapy.

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