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Presence of Hepatitis B virus DNA in follicular fluid in female Hepatitis B carriers and outcome of IVF/ICSI treatment: A prospective observational study

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ABSTRACT

Objective: To determine the relationship between the presence of detectable HBV DNA in the follicular fluid in HBV carriers with IVF/ICSI treatment outcome.
Study design: A prospective observational study conducted in the Assisted Reproductive Unit, a tertiary referral centre affiliated with the Department of Obstetrics and Gynecology, The Chinese University of Hong Kong; and the Union Reproductive Medicine Centre at Union Hospital, Hong Kong. The primary outcome measure was pregnancy rate. Secondary outcome measures were the prevalence of detectable HBV DNA in the follicular fluid, implantation rate, clinical pregnancy rate, ongoing pregnancy rate and live birth rate.
Results: HBV DNA was detected in the follicular fluid of 28 (43.8%) of the 64 women, and the mean level in this group in $\log_{10}$ copies/mL (±SD) was 4.36 ± 1.85. Women with detectable follicular fluid HBV DNA were younger, lighter, had longer duration of infertility, higher incidence of detectable serum HBV DNA (OR 4.592, 95% C I 2.333–9.038), and significantly wider range in the number of total fertilized, viable embryos, and blastocyst rate, but no difference in cycle characteristics, stimulation and pregnancy outcomes, although the almost doubled ongoing pregnancy/live birth rate per cycle initiated (60.7% versus 38.9%) failed to reach statistical significance due to the small numbers.
Conclusion: Our results suggested HBV infection did not appear to be detrimental to the outcome of IVF/ICSI treatment.

Introduction

Chronic Hepatitis B virus (HBV) infection is endemic worldwide, and the prevalence is especially high in the Asia-Pacific regions and immigrants from these regions [1–3]. The prevalence is as high as 8–10% or more among both pregnant women at antenatal screening [4] as well as infertile women seeking in-vitro fertilization/ intracytoplasmic sperm injection (IVF/ICSI) treatment in Hong Kong [5,6]. Despite its high prevalence, the impact of HBV infection on fertility and fertility treatment remains unclear. Indeed, it has also been shown that in women undergoing their first IVF/ICSI cycles, both the ongoing pregnancy rate or live birth rate and implantation rates were significantly higher in women with HBV infection compared with non-infected women (53.3% vs. 24.2% per cycle with embryo transfer; and 43.3% vs. 18.4%, respectively) [5]. Nevertheless, there were other studies that found that the IVF/ICSI outcome did not seem to be affected [6–8] with similar pregnancy rates between HBV seropositive and seronegative women [6], and one study even found a lower pregnancy rate [9].

In men, HBV infection has been associated with poor semen parameters [7,10] and reduced reproductive capacity [11]. In women with HBV infection, the virus has been found in the ovarian tissue including the ova, and granulosa and other cells [12–17]. Yu et al studied HBV covalently closed circular DNA (cccDNA) expression, which reflects the status of virus-active replication, in 33 human ovarian tissue samples, and found that 12 and 14 samples had HBV DNA and HBV cccDNA respectively, thus demonstrating the capability of HBV to replicate in the ovary (24). Of note, the level of both HBV DNA and HBV cccDNA were

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significantly lower than the corresponding serum HBV DNA levels [17]. In another study, Kong et al examined HBV expression and replication using HBV mRNA in the ova of 50 HBV carriers and found three ovarian tissue samples were positive for HBV mRNA, which was mainly distributed in the cytoplasm of the ova and granulosa cells, and its presence was seen in the primary ova in one case [16]. Therefore, it is possible that infected ovaries or oocytes exhibit a different response to ovarian stimulation during IVF/ICSI treatment, have different rates of fertilization, embryogenesis, and possibly implantation, and last but not least, would be one of the vehicles for vertical transmission of HBV to the offspring [15].

A prospective study was therefore conducted on infertile women seeking IVF/ICSI treatment who are screened to have HBV infection to determine the incidence of detection of HBV DNA in the follicular fluid as a surrogate marker since it is unethical to sacrifice the oocytes of these patients, and the relationship between the presence or absence of detected HBV DNA on IVF/ICSI treatment outcome.

Materials and methods

Study design

This was a prospective observational study conducted in the Assisted Reproductive Unit of the Department of Obstetrics and Gynaecology of The Chinese University of Hong Kong, and the Union Reproductive Medicine Centre at Union Hospital, Hong Kong, during the period of September 2013 to August 2017.

Study population

Women who were scheduled IVF procedure and screened to be HBV carriers were invited to participate in this study with written consent. The exclusion criteria included (i) the male partner also had HBV infection; (ii) women who were symptomatic or with clinical manifestations of HBV infection; (iii) the presence of other blood borne infections including Hepatitis C, HIV and syphilis.

Procedure

Each woman underwent only a single ovarian stimulation and oocyte retrieval cycle as per protocol. The follicular fluid obtained from the aspiration of the first follicle during oocyte retrieval was used for the analysis of the HBV DNA viral load. The follicular fluid obtained from subsequent follicles in the same ovary was not used for analysis because of the possibility of blood contamination from repeated follicle puncture. The oocyte was isolated from the follicular fluid and underwent IVF/ICSI with the partner's sperm in the laboratory. The follicular fluid and cumulus cells from the first follicle aspirated from each ovary were, instead of being discarded as in usual clinical practice, stored at 4 °C for measurement of HBV DNA level later. A paired peripheral blood sample (10 ml) was collected at the same time and stored at 4 °C for detection of hepatitis B surface antigen (HBsAg) and hepatitis B envelope antigen (HBeAg), and serum HBV DNA level.

Outcome measures

The primary outcome measure was pregnancy rate (PR), which was defined as positive hCG per embryo transfer. Secondary outcome measures were implantation rate (IR) which was defined as the number of gestational sacs seen on ultrasound examination per embryo transferred, clinical pregnancy rate (CPR) which referred to the number of pregnancies with confirmed intrauterine gestational sac, ongoing pregnancy rate (OPR) which referred to the presence of at least one foetus with heart pulsation proceeding beyond 32 weeks of gestation [18] and live birth rate (LBR) which referred to the number of deliveries with at least one live born infant.

Sample size

In one study, PR for couples with HBV undergoing IVF cycles was reported to be significantly reduced to 7.7% when compared with 40.7% of age-matched control subjects [9]. Two studies in Hong Kong reported that PR varied from 23% [6] to 50% [5]. The underlying reason for the marked difference of PR among HBV carriers is unclear. We hypothesize that the DNA viral load has a significant impact on the outcome. There is no literature data to suggest if the HBV DNA titre in follicular fluid has any impact on IVF/ICSI outcome and to provide guidance on sample size calculation. Nevertheless, we estimated that a sample size of 60 should be sufficient for a pilot, observational study to be conducted.

Ethics approval

The study was approved by the Joint Chinese University of Hong Kong – New Territories East Cluster Clinical Research Ethics Committee on 17th April 2013 (CREC Ref. No.: 2013.108) and Union Hospital's Ethics Committee on 11th September 2015 (Ref. No.: LET-DMD037-15).

Table 1
Baseline characteristics between subjects with and without detectable HBV DNA in follicular fluid.

	HBV DNA positive (n = 28)	HBV DNA negative (n = 36)	*P*
Age (years)[a]	34.79 ± 2.41	36.42 ± 3.21	0.029
BMI (kg/m^2)[a]	20.66 ± 1.77	22.08 ± 2.76	0.021
Infertility duration (years)[a]	6.96 ± 3.57 5	5.17 ± 3.48	0.047
Previous pregnancy loss (%)[b]	8 (28.6)	12 (33.3)	0.683
Previous live birth (%)[b]	4 (14.3)	7 (19.4)	0.743
Type of infertility (%)[b]			
Primary	15 (53.6)	13 (36.1)	0.182
Secondary	13 (46.4)	23 (63.9)	
Cause of infertility (%) [b,c]			
Tuboperitoneal	12 (42.8%)	14 (40%)	
Endometrosis	2 (7.1%)	5 (13.9%)	
Male	15 (53.6%)	21 (58.3%)	0.762
Unexplained	1 (3.6%)	3 (8.3%)	
Serum HBV DNA detected (%)[b]	25 (89.3)	7 (19.4)	<0.001

[a] Data expressed in mean ± SD and comparison using Student's t-test.
[b] Data expressed in number (%) and comparison using χ^2 test# or Fisher's exact test where appropriate.
[c] Some patients presented with more than one cause of infertility.

Statistical analysis

All analyses were performed using the Statistical Package of Social Sciences for Mac Version 23 (IBM Corp., USA). Continuous data were assessed for normality of distribution using the Shapiro–Wilk test, and expressed as mean (±SD) or median (interquartile range) accordingly. Normally distributed data was compared using the Student's T-test. For non-normally distributed data, the Mann–Whitney U test was used to compare distribution across groups, while the Moses Test of Extreme Reaction was used to compare the ranges, some of which were very wide, across groups. Categorical data were expressed as number (percentage); comparisons were made using the X^2 test or Fischer's exact test and presented with rate ratios and 95% CI. A two-sided value of $P < 0.05$ was considered as significant.

Results

Baseline characteristics of participants

A total of 64 female HBV carriers were assessed for eligibility and recruited into the study. HBV DNA was detected in the follicular fluid of 28 (43.8%) of them, and the level of HBV DNA expressed in mean, median, and interquartile range (IQR) in $\log_{10}$ copies/mL was 4.36, 4.45, 2.76–6.01 respectively. Women with detectable follicular fluid HBV DNA were significantly younger, lighter, and had a longer duration of infertility (Table 1). There was however no difference in the incidence of primary or secondary infertility. Nevertheless, women with detectable follicular fluid HBV DNA had a significantly higher incidence of detectable serum HBV DNA (OR 4.592, 95% C I 2.333–9.038).

Response to IVF treatment and cycle characteristics

The results of ovarian stimulation in the two groups are compared in Table 2. There was no significant difference in the stimulation or ovarian response between the two groups. However, significant difference was found in the ranges of total oocytes fertilized (Moses Test $P = 0.032$), IVF total fertilized (Moses Test $P = 0.003$) as well as ICSI total fertilized (Moses Test $P = 0.013$), while the overall fertilization rates were similar between the two groups (66.78 ± 19.46% vs 65.98 ± 23.64% in follicular fluid HBV DNA positive and negative group respectively, $P = 0.924$). Interestingly, more viable embryos were noted in follicular fluid HBV DNA positive group (3 vs 2 in follicular fluid HBV DNA positive and negative group respectively, $P = 0.013$), however after matching with age, there was no statistically significant difference between the two groups. Besides, significant difference was found in the range of blastocyst rate as well (Moses Test $P < 0.001$).

Outcome analysis

The pregnancy outcome analysis of the study is presented in Table 3. There was no significant difference in PR and IR between the groups. Although the OPR/LBR per cycle initiated was almost doubled in the HBV DNA positive group, the difference failed to reach statistical significance due to the small numbers. Multivariate analysis was performed by binary logistic regression to take into consideration of BMI, age and duration of infertility as there were significance difference between the groups with and without HBV DNA in the follicle fluid. And there were no statistical difference in the pregnancy outcomes. (Pregnancy rate: OR 1.319, 95% CI 0.397–4.384; clinical pregnancy rate per cycle initiated: OR 1.277, 95% CI 0.401–4.060; ongoing pregnancy/live birth rate per cycle initiated OR 1.297, 95% CI 0.414–4.062).

Discussion

To the best of our knowledge this is the first prospective observational study to examine the impact of HBV infection in the ovarian follicles, as represented by the detectable HBV DNA in follicular fluid, on the outcome of IVF pregnancy in chronic HBV female carriers. We found that among women screened to be HBsAg positive and intended to undergo IVF treatment, HBV DNA can be detected in as much as 44%. This group of women had slightly but significantly different characteristics, and although younger, they also had a longer history of infertility, but there was no difference in the incidence of primary or secondary infertility, or in the causes of infertility. The higher incidence of detectable serum HBV DNA in the women with detectable follicular HBV

Table 2
Ovarian stimulation response in subjects with and without detectable HBV DNA in follicular fluid.

	HBV DNA positive (n = 28)	HBV DNA negative (n = 36)	*P value for distribution*	*P value for range*
Antral follicle count (pre-treatment)[b]	9.18 ± 3.03 (n = 17)	7.67 ± 3.41 (n = 21)	0.083	0.094
Antral follicle count (treatment)[b]	8.43 ± 4.23 (n = 23)	8.23 ± 3.48 (n = 31)	0.860	0.668
Number of follicles ≥15 mm[b]	6.82 ± 3.72	6.61 ± 3.30	0.875	0.890
Number of follicles <15mm[b]	5.20 ± 2.52	5.27 ± 3.52	0.615	0.685
Total oocytes retrieved[b]	9 (7-14)	8 (5.25-12.75)	0.597	–
Total mature oocytes retrieved[b]	8.5 (4.25-12)	7.5 (4-10.75)	0.640	–
Insemination method[a]				
Conventional	10 (35.7%)	15 (41.7%)	0.234	–
ICSI	18 (64.3%)	21 (58.3%)		
Total fertilized[b]	6.75 ± 4.11	6.58 ± 5.00	0.532	**0.032**
IVF total fertilized[b]	3.22 ± 3.98 (n = 23)	2.68 ± 3.85 (n = 31)	0.755	**0.003**
ICSI total fertilized[b]	4.26 ± 4.15 (n = 27)	4.28 ± 5.63 (n = 36)	0.537	**0.013**
Fertilization rate (%)[b]	66.48 ± 19.46	65.98 ± 23.64	0.924	0.380
Viable embryos [b]	3 (2-5)	2 (1-4)	**0.013**[c]	–
Blastocyst rate[b]	37.00 ± 44.80 (n = 28)	34.80 ± 46.85 (n = 34)	0.736	<**0.001**
Embryo transfer[a]				
Fresh	17 (60.7%)	21 (58.3%)	0.673	–
Frozen	11 (39.3%)	14 (38.9%)		
Subjects with more than one embryo transfer cycle arising from one oocyte retrieval (%)[a]	9 (32.1%)	8 (22.9%)	0.569	–

[a] Data expressed in number (%) and comparison performed with the χ^2 test# or Fisher's exact test where appropriate.
[b] Data expressed in mean ± SD, median (interquartile range); comparison performed with Moses Test of Extreme reaction for range/Mann Whitney U test for distribution.
[c] After age matched, there was no statistically significant difference between the two groups.

Table 3
Pregnancy outcome per transfer among subjects with and without detectable HBV DNA in follicular fluid.

	HBV DNA positive (n = 28)	HBV DNA negative (n = 36)	*p*
Total number of embryos transferred[a]	58	65	–
Total number of embryo transfer cycles[a]	40	44	–
Pregnancy rate (%) #	21/40 (52.5%)	23/44 (52.3%)	0.983
Implantation rate (%) #	23/58 (39.7%)	24/65 (36.9%)	0.756
Clinical pregnancy per cycle initiated (%) #	19/28 (67.9%)	20/36 (55.6%)	0.317
Clinical pregnancy per transfer (%) #	19/40 (47.5%)	20/44 (45.5%)	0.851
Ongoing pregnancy/ Live birth rate per cycle initiated (%) #	17/28 (60.7%)	14/36 (38.9%)	0.083
Ongoing pregnancy/ Live birth rate per transfer (%) #	17/40 (42.5%)	14/44 (31.8%)	0.310

Values are expressed as number (percentage), and analyzed by chi-square test#.
[a] Including both fresh and frozen embryos transferred.

DNA suggested that they had more active HBV activity, and that this group may be especially hazardous to the laboratory staff handling their samples. As well, women with detectable HBV DNA in the follicular fluid exhibited a slightly higher range of total oocytes, and significantly higher range of total fertilized, including both IVF and ICSI total fertilized, even though as a group the overall distribution of the results was not significantly different. Finally, the women with detectable follicular fluid HBV DNA showed a trend towards a higher OPR/LBR rate per cycle initiated, although no difference in outcome was found which was probably related to the small number of cases due to the lack of information to allow the estimation of an adequate sample size. While the differences did not reach statistical significance, our results nevertheless did suggest that the follicles and oocytes with evidence of HBV infection appear to behave differently, and higher OPR/LBR per cycle observed was in line with the previous study that both OPR/LBR and IR were significantly higher in women with HBV infection compared with non-infected women (53.3% vs. 24.2% per cycle with embryo transfer; and 43.3% vs. 18.4%, respectively) [5].

Clinical outcome in women with HBV infection

Previous studies evaluating the impact of HBV infection in couples with at least one partner being HBV seropositive and undergoing the first cycle of IVF treatment outcome yielded conflicting results [5,6,9,19]. The study by Pirwany et al reported lower pregnancy rate (7.69% in 13 couples discordant for HBV vs 41% in 27 controls) [9], while Lam et al found a significant higher OPR or LBR among 56 couples who were discordant for HBV (53.3% vs 242% per cycle with embryo transfer; and 43.3% vs 18.4% respectively) [5]. The study by Shi et al found no difference in CPR between women who were HBsAg seropositive and seronegative (48.1% vs 50.6%) [19] and Lee et al examining 131 women who were HBV seropositive with and without male partner being HBV seropositive suggested that OPR was similar (26.7% vs 30.2% per cycle initiated; 34% vs 33.8% per transfer respectively) [6]. Yet in these studies, either the female and/or the male partner were HBV carriers, the possible effect of HBV infection on the male partner in treatment outcome was therefore an important confounding factor since there is evidence suggesting that HBV infection in the male partner is associated with poor sperm parameters and lower fertilization rate, although there appeared to be no negative impact on IVF/ICSI [6–8]. The only published study examining 123 HBsAg seropositive women with seronegative male partners undergoing IVF/ICSI treatment reported that chronic HBV infection was not associated with pregnancy outcomes (CPR 44.72% vs 43.09%; IR 30.52% vs 28.34%; LBR 42.28% vs 40.65%) [20].

Previous studies also revealed that HBV DNA can be found in the ovarian tissue including ova, granulosa and other cells [12–17], so that there is the possibility of an effect on replicating capacity [16,17]. However, Feng et al studied 38 abandoned single cell cleavage embryos from 14 HBsAg seropositive women with seronegative male partners, and found no difference in the CPR (35.7% vs 44%). But studying embryos would involve either abandoned embryos, which are likely to be of poor quality which might or might not be due to HBV infection, or sacrificing good embryos that could be replaced into the uterine cavity. In order to avoid any selection bias or ethical dilemma, we took follicular fluid HBV DNA as the surrogate marker of HBV infection in the oocyte for all women with chronic HBV infection, and examined only the follicular fluid from the first aspiration to avoid misinterpretation due to contamination. Overall, we could not demonstrate increased adverse outcome in the women with HBV activity in the ovarian follicles. On the other hand, the result actually showed a trend that was in line with the study of Lam et al [5], but our sample size was insufficient to be conclusive on this issue.

The adopted standard strategy to prevent vertical transmission of HBV from the mother to the offspring involved HBV vaccination and Hepatitis B immunoglobulin administered shortly after birth [21–24], yet immunoprophylaxis failure can occur in as high as 5–10% of the offspring [25–27], and vertical transmission via human germ cells could be one of the mechanisms. Hu et al showed the presence of HBV DNA in human oocytes or embryos in seropositive women (9.6% and 14.4% respectively) and the rate of positivity were significantly higher in group with high serum HBV level [28]. Another study by Yu et al, exploring the HBV infection in ovarian follicles on its vertical transmission, found 3 infants to be HBV positive out of 7 infants whose mothers had HBsAg in the ovaries (42.85%) and both mothers who had HBV infected oocytes gave birth to intrauterine infected infants [15]. Our result suggests that less than 50% of women seeking IVF treatment who are seropositive for HBsAg exhibited HBV activity in the ovarian follicles, so that HBV infection in the oocyte could have been one of the overlooked factors that could have accounted for vertical transmission despite immunoprophylaxis. Further studies are warranted to clarify this issue.

Conclusion

In our population, 44% of women seeking IVF treatment, who were seropositive for HBsAg, showed evidence of HBV infection in their oocytes, which however did not appear to be detrimental to the IVF/ICSI treatment's outcome. Nevertheless, further studies are warranted to determine if this represents another mechanism of vertical transmission to the offspring, for which antiviral medication may be indicated following successful IVF/ICSI treatment.

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Conflicts of interest

The authors have no conflicts of interest to disclose.

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