



















Hepatitis B Surface Antigen Seropositive Men in Serodiscordant Couples: Effects on the Assisted Reproductive Outcomes

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Purpose: To evaluate the influence of hepatitis B virus (HBV) infection in men of serodiscordant couples on the reproductive outcomes.

Materials and Methods: A total of 134 infertile couples were included in this retrospective single-center cohort study. Sixty-six couples had hepatitis B surface antigen (HBsAg)-seropositive men and seronegative partners, while 68 couples were controls with both seronegative men and women. Overall, 134 fresh *in-vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) treatments were performed. As the main outcome measures, on the day of the fresh IVF/ICSI cycle, we assessed seminal parameters Before and after sperm preparation techniques. Two-pronuclear (2PN) fertilization, 1-2-3PN fertilization, cleavage, miscarriage, pregnancy and live birth rates were collected.

Results: No significant differences were found between groups in terms of oocytes retrieved, oocytes injected and embryos obtained ($p=0.64$, $p=0.97$, and $p=0.40$, respectively). The 2PN fertilization rate (FR) was comparable among groups ($p=0.51$). The 1-2-3PN FR was significantly lower in the HBsAg group than in the control group (66.6% vs. 69.7%, respectively). The clinical pregnancy per cycle, implantation, miscarriage and live birth rate were comparable between the HBsAg group and the control group. The median sperm concentration/ml and total sperm count, measured at baseline and after sperm preparation, was comparable between groups ($p>0.05$). There was a trend toward significant lower progressive motility (35.0% vs. 55.0%; $p<0.05$) in the HBsAg group at baseline and after sperm preparation ($p<0.05$).

Conclusions: HBV infected men have the same chance to become father, compared to seronegative patients.

Keywords: Anti-hepatitis B antigens; Hepatitis B virus; Infection; Intracytoplasmic sperm injections; *In Vitro* fertilization; Male infertility

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INTRODUCTION

Hepatitis B virus (HBV) is considered one of the most widespread blood-borne infections, interesting about two billion people in their life [1]. Usually, HBV disease, described as the evidence of serum hepatitis B surface antigen (HBsAg) [2], leads to hepatitis, cirrhosis and hepatocellular carcinoma [3]. Nevertheless, HBV has been detected in several organs, including kidney, parotid glands, ovaries [4] and testes [5], as well as in seminal fluid [6].

Since it has been demonstrated that HBV could raise chromosomal instability in spermatozoa [7], causing damage of sperm viability and normal morphology [8], the correlation between HBV infection and male fertility should be considered a remarkable current issue. In this respect, a growing amount of HBV-infected men with a history of couple infertility resort to assisted reproductive technologies (ARTs), such as *in-vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) treatments. Moreover, the probability of viral transmission to the woman or fetus, clinical or embryological issues and cross-infection of other virus-free gametes or embryos raised concerns. In fact, HBV infection can result in vertical transmission to the newborn by inserting sperm and mixing into the genome of the embryo [9].

In this scenario, some studies stated that HBV mRNA was identified in the embryos of HBV-infected fathers, implying the transcription and replication of HBV genes [10,11]. Nevertheless, while previous authors showed significantly lower pregnancy rate (PR) in HBV discordant couples likened to age-matched controls [12], other ones detected higher pregnancy, live birth and implantation rates (IRs) in HBV group [13]. On these bases, a general consensus about the influence of the disease on final reproductive findings in seropositive couples lacked.

Therefore, the purpose of the study is to evaluate the influence of HBV men infection on IVF/ICSI outcomes, in a cohort of consecutive serodiscordant couples (SDCs).

MATERIALS AND METHODS

1. Patients selection

From January 2011 to August 2018, a retrospective single-center cohort study of infertile couples referring to our ARTs Centre, for the first ART treatment,

was carried out. Inclusion criteria were: 1) male age range between 18 and 45 years and female age range between 18 and 40 years; 2) HBV-SDCs, in which the men was HBsAg seropositive and the women seronegative. When the patients presented with positive HBsAg measured in the serum *via* enzyme immunoassay for at least 6 months were defined as chronic HBV carriers. Exclusion criteria were: 1) the presence of abnormal liver function or chronic hepatitis; 2) clinical presentation of azoospermia or severe cryptozoospermia; 3) cycles with donor's semen or chromosomal aberrations; 4) couples who were seropositive for hepatitis immunodeficiency virus (HIV) and/or hepatitis C virus (HCV). Additional exclusion criteria were: 1) positive history of parotitis; 2) antiviral therapy during the study period.

2. Cause of couple infertility and laboratory assessment

The cause of couple infertility was categorized into 5 groups: 1) tubal factor; 2) endometriosis; 3) male factor; 4) unexplained; 5) mixed. Controls included both men and women who were negative for serum HBsAg, hepatitis B surface antibody (HBsAb), hepatitis B e antigen, hepatitis B e antibody, and hepatitis B c antibody (HBcAb). Control couples were also matched for age, ART approach used (IVF or ICSI) and cause of infertility.

Both partners were evaluated by an interdisciplinary specialized fertility team. On the second day of the cycle, all women underwent endocrinology assessment, comprising follicle stimulating hormone (FSH), luteinizing hormone, estradiol, anti-mullerian hormone, thyroid-stimulating hormone, prolactin. All men were examined for a complete physical and andrological visit. Baseline features, including male and female's age, were collected. A dedicated infectious disease specialist screened both male and female for HIV 1/2, HCV antibody, HbsAg, HbsAb, HBcAb, Treponema Pallidum Hemagglutination and Venereal Disease Research Laboratories, Ab anti-Clamidia Trachomatis and Ab anticytomegalovirus.

All the men enrolled performed semen analysis, evaluated according to the 2010 World Health Organization (WHO) recommendations. At the time of fresh IVF/ICSI treatment, we measured seminal characteristics, as follow: volume, pH, total sperm count/mL, total sperm concentration, viability, progressive motility (PR), non-PR and normal morphology. After sperm

capacitation, we analysed total sperm count/mL and motility.

3. Sperm preparation techniques

Semen samples were obtained by masturbation after 2 to 7 days of sexual abstinence and collected into sterile plastic containers. After liquefaction in the incubator set at 37°C for 30 minutes, ejaculates were analyzed and classified (WHO, 2010). Semen samples from HBV-infected men were processed under sterile conditions using the density gradient centrifugation method with 95%, 70% and 50% gradient layers (PureSperm[®]100; Nidacon Mölndal, Sweden); heated in the incubator set at 37°C for 30 minutes. Removing the supernatant at each step before the pellet transfer helped to minimize any viral transmission. Subsequently, 1 mL of 95% gradient, 1 mL of 70% gradient, and 1 mL of 50% gradient were gently overlaid in a tube: finally, 1 mL of the sperm was gently overlaid on the 50% gradient and centrifuged at 300×g for 20 minutes. The supernatant was carefully aspirated and discarded using a sterile Pasteur pipette for each tube. The pellet was moved into a new conical tube, re-suspended in 2.5 mL of sperm medium (Flushing; Origio, CooperSurgical Fertility & Genomic Solutions, Måløv, Denmark) and centrifuged again at 250×g for 10 minutes. After centrifugation, the supernatant was discarded using a disposable sterile Pasteur pipette and was re-suspended in 1 mL of sperm medium (Fert; Origio, CooperSurgical Fertility & Genomic Solutions).

Sperm count and motility assessment were then performed on the washed pellet under sterile conditions. This pellet was stored for later use in the ART procedure.

4. Ovarian stimulation protocol and assisted reproductive technology treatment

Treatment individualization is based on ovarian response prediction, based on the Anti-Müllerian hormone and antral follicle count. The initial prescription of recombinant FSH was 225 to 375 IU (Gonal-F; Merck, Geneva, Switzerland), depending on age and baseline blood FSH measure. Follicular growing was monitored and the FSH dosage was adjusted based on the follicular response. When follicles ≥ 14 appeared, gonadotropin-releasing hormone antagonist was used. Follicular development was checked through frequent ultrasounds to choose the more appropriate phase

of oocytes recovery. When at least two follicles had developed a maximal diameter of 17 to 18 mm, an administration of 250 µg recombinant human chorionic gonadotropin (HCG; Ovitrelle; Merck) was subcutaneously administered. Transvaginal oocyte recovery was performed approximately 36 hours later. All oocytes picked by HBV SDCs were accepted for IVF or ICSI treatment, according to seminal parameters. The incubation of injected oocytes was done in 20 µL drops. All the zygotes were valued 16 to 18 hours by ICSI to confirm the presence of two distinct pronuclei (2PN). Then, all the embryos were evaluated on days 2, 3, and 5 of the progress with an inverted microscope.

5. Assisted reproductive technologies outcome measures

IVF/ICSI results, as well as 2PN fertilization rate (FR), 1-2-3PN FR, and cleavage rate (CR) were recorded. Total and normal oocyte FR was obtained by total number of fertilized oocytes (1-2-3 PN) and 2PN fertilized oocytes by the number of injected oocytes, respectively. The CR was considered by the number of embryos obtained by the number of 2PN fertilized oocytes. The luteal phase was maintained by a daily administration of 50 mg intramuscularly of natural progesterone (Prontogest; Amsa, Rome, Italy), since the day of oocytes retrieval. Embryo transfer was performed on day 3 to 5 after IVF/ICSI procedure, using a Wallace catheter (©COOK Medical Incorporated, Bloomington, IN, USA). Supernumerary embryos are frozen, according to our laboratory policy. After 14 days, the HCG test was done. The IR was expressed as the number of gestational sacs per embryo transferred. Clinical PR per cycle was calculated by cycles with HCG levels above 50 mU/L and confirmed by transvaginal ultrasounds revealing an intrauterine gestational sac with a heartbeat at around 5 to 6 weeks of gestation. Pregnancy loss before 20 weeks of gestation and all biochemical pregnancies were contemplated as miscarriages. Live birth rate was defined as the percentage of all cycles that lead to live births.

6. Ethical statement and statistical analysis

All procedural protocols were permitted by the Institutional Review Board (IRB) of University of Florence, Careggi Hospital (IRB No. CS/1158/04.1). All couples provided written informed consent before the start of ART treatment.

Non-parametric continuous variables, count, and proportion distributions were compared between groups applying the non-parametric unpaired Wilcoxon rank-sum test with continuity correction or the Fisher exact test. The G*Power program was used to calculate the sample size needed for the abovementioned statistical tests. In particular, the asymptotic relative efficiency method was used to estimate the power for the Wilcoxon rank-sum test. Analyses based on an intention-to-treat approach and considered a statistically significant level of $p < 0.05$ (2-sided). Data analysis was performed using R ver. 3.3.0 (R Development Core Team) and data management was performed using SAS statistical software ver. 9.4 (SAS Institute Inc., Cary, NC, USA).

RESULTS

This retrospective cohort study included 134 infertile

Table 1. Demographic and laboratory data of HBsAg-positive patients and controls

Parameter	HBsAg (n=66)	Controls (n=68)	p-value
Male age (y)	37.0 (32.2–41.0)	38.0 (36.0–42.0)	0.51
Female age (y)	34.0 (29.2–36.0)	36.0 (34.0–39.0)	0.46
Cause of infertility			
Tubal factor	20 (30.3)	24 (35.3)	0.41
Endometriosis	14 (21.2)	14 (20.6)	0.46
Male factor	22 (33.3)	23 (33.8)	0.67
Unexplained	5 (7.6)	4 (5.9)	0.34
Mixed	5 (7.6)	3 (4.4)	0.45
Oocytes retrieved (n)	6.0 (2.0–7.0)	5.0 (3.0–7.0)	0.38
Oocytes inseminated (n)	4.0 (3.0–6.0)	4.0 (3.0–6.0)	0.46
Oocytes 2PN fertilized (n)	3.0 (2.0–4.0)	2.0 (2.0–5.0)	0.58
Oocytes total 1-2-3PN fertilized (n)	3.0 (2.0–4.0)	3.0 (2.0–5.0)	0.51
Embryos obtained (n)	2.0 (1.0–3.0)	2.0 (1.0–4.2)	0.46
Embryos transferred (n)	2.0 (1.0–2.0)	2.0 (1.0–2.0)	0.46
Fertilization rate 2PN (%)	66.6 (50.0–75.0)	66.6 (50.0–80.0)	0.51
Fertilization rate 1-2-3PN (%)	66.6 (50.0–70.0)	69.7 (50.0–90.2)	0.03*
Cleavage rate (%)	100.0 (52.5–100.0)	100.0 (100.0–100.0)	<0.001*

Values are presented as median (interquartile range) or number (%). HBsAg: hepatitis B surface antigen, PN: pronuclear.

*Statistical significance ($p < 0.05$).

couples: 66 couples had HBsAg-seropositive men and 68 were controls. Overall, 134 fresh ART treatments were performed: 95 ICSI cycles and 39 IVF cycles. In the HBsAg group, 17 couples had IVF, while 49 had ICSI. In the control group, 22 couples had IVF and 46 had ICSI. Demographic and laboratory data of the HBsAg-positive patients and controls are presented in Table 1. Patients' baseline characteristics did not significantly vary between the HBV-positive and HBV-negative groups. Overall, the cause of couple infertility was: tubal factor (32.8%), endometriosis (20.9%), male factor (33.6%), unexplained (6.7%), mixed (6.0%). No significant differences were found between groups in terms of oocytes retrieved, oocytes injected and embryos obtained ($p=0.64$, $p=0.97$ and $p=0.40$, respectively). 2PN FR was comparable among groups ($p=0.51$). As shown in Fig. 1, total FR and CR were significantly lower in the HBsAg group than in the control group ($p=0.03$; $p<0.001$, respectively). Women in the two groups were transferred with similar number of embryos (median=2.0; interquartile range=1.0–2.0). Pregnancy outcomes are depicted in Table 2. The clinical pregnancy per cycle, implantation, miscarriage and live birth rate were comparable between the HBsAg group and the control group. As shown in Fig. 2, the clinical PR was not statistically different between groups after adjusting for confounding variables (odds ratio=1.28, 95% confidence interval=0.57–2.95, $p=0.56$).

The live births, summing singleton and twin pregnancies, for couples with HBsAg-positive man and controls were respectively 16 and 19.

Seminal characteristics of the two groups at baseline and after sperm preparation techniques are summarized in Table 3. The median sperm concentration/ml and total sperm count, measured at baseline and after sperm preparation, was comparable between groups, suggesting that sperm production was not affected by the HBV infection ($p > 0.05$). There was a trend toward significant lower PR (35.0% vs. 55.0%; $p < 0.05$) in the HBsAg group at baseline. Equally, total motility remained significantly lower after sperm preparation in the HBsAg group ($p < 0.05$).

DISCUSSION

Most of the available evidences evaluating the role of HBV infection on fertility derive from areas in which the virus is endemic [1].

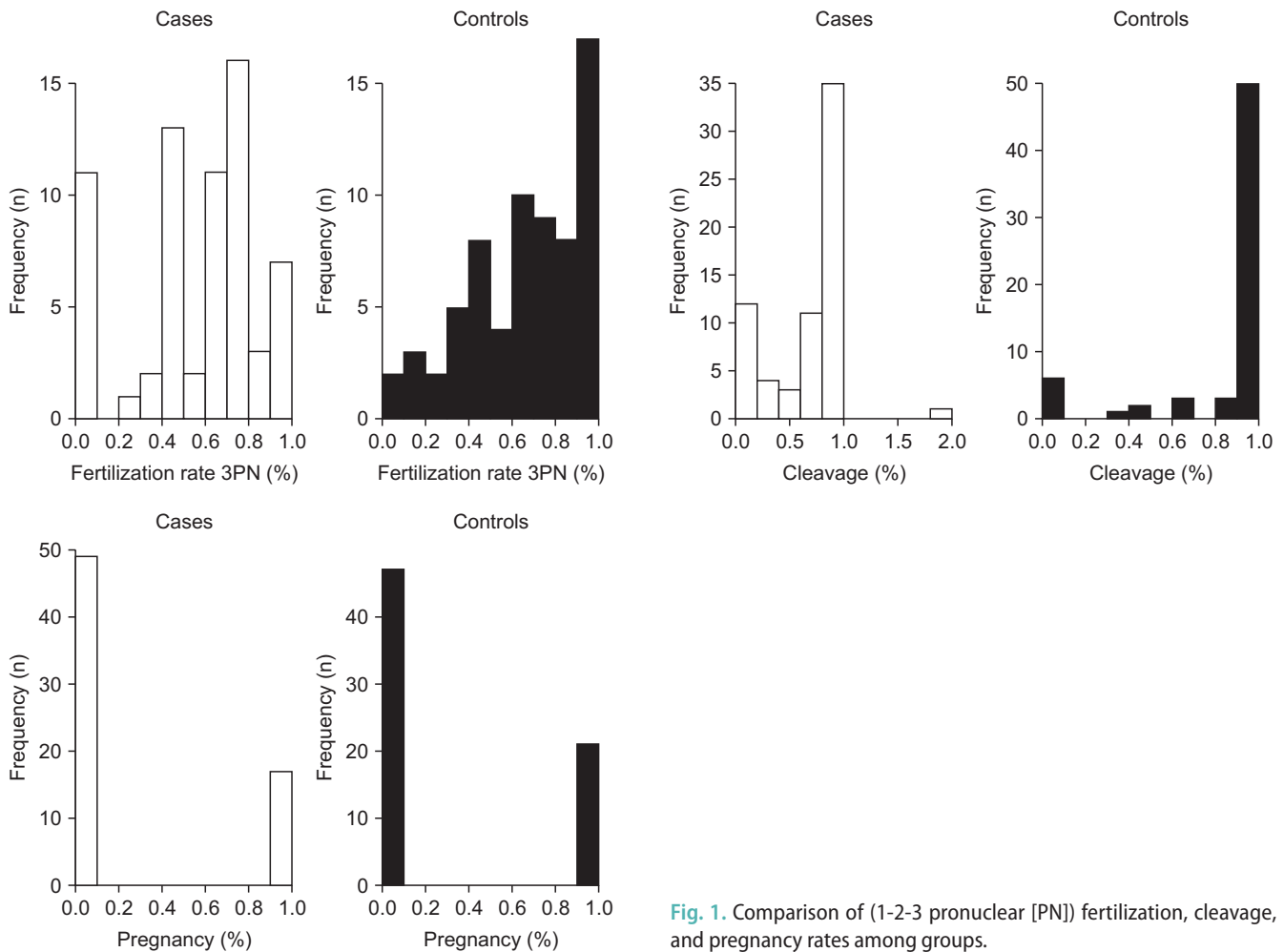


Fig. 1. Comparison of (1-2-3 pronuclear [PN]) fertilization, cleavage, and pregnancy rates among groups.

Table 2. Pregnancy outcomes

Variable	HBsAg	Controls	p-value
Implantation rate	20/58 (34.5)	25/99 (25.3)	0.78
Pregnancy rate per cycle	17/66 (25.8)	21/68 (30.9)	0.56
Miscarriage rate per cycle	3/17 (17.6)	7/21 (33.3)	0.32
Live birth rate per cycle	14/66 (21.2)	13/68 (19.1)	0.83

Values are presented as number/total number (%).

In our center, screening for HBV before the ART program is a relevant part of our enrolment schedule. Consequently, there is no ethical motivation to refuse ART treatments in HBsAg seropositive men, albeit in zones little endemic for HBV disease.

The rationale of our research is the knowledge that HBV has the capacity of penetrating the blood-testis barrier and integrating into human sperm chromosomes [2]. In this way, the virus could induce mutagenic effects, which could affect the embryological and clinical outcomes of pregnancy. In this scenario, the

influence of men HBV infection on fertility represents an important topic, since any anomalies from germ cells could affect assisted reproductive outcomes, like fertilization, implantation, and pregnancy. However, since very conflicting results exist in literature, this work aimed to clarify this issue as much as possible, to achieve a general consensus.

Previous clinical retrospective studies analyzed the implication of viral infection on the seminal parameters, but none of them reached a common conclusion. Some researchers detected that, when compared with the control group, HBV seropositive men had lower sperm motility and total sperm count, as well as poor morphology [14,15].

However, in our study sperm concentration/mL and total sperm count appear similar between groups, also after sperm preparation, suggesting that the viral infection not influence spermatogenesis. Equally, semen volume and normal sperm morphology of HBsAg group were comparable with controls, in line with other stud-

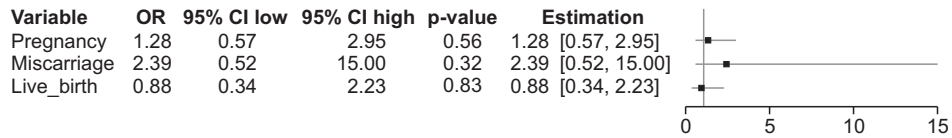


Fig. 2. Comparison of pregnancy, miscarriage and live birth rates among groups, after adjusting for confounding variables. OR: odds ratio, CI: confidence interval.

Table 3. Seminal characteristics at baseline and after sperm preparation techniques

Parameter	HBsAg (n=66)	Controls (n=68)	p-value
Volume (mL)	3.1 (1.2–4.4)	2.8 (1.2–4.8)	0.51
pH	7.6 (7.4–7.8)	7.8 (7.4–8.0)	0.46
Viability (%)	71.5 (58.0–78.0)	72.0 (66.0–78.0)	0.35
Concentration (sperm/mL)	31.5 (9.2–62.2)	22.5 (6.7–60.5)	0.42
Total sperm count	90.0 (23.4–228.4)	87.2 (15.8–187.9)	0.26
Progressive motility (%)	35.0 (16.2–45.0)	55.0 (40.0–62.7)	<0.001*
Non-progressive motility (%)	10.0 (10.0–20.0)	5.0 (5.0–10.0)	<0.001*
Morphology (%)	6.0 (4.0–6.2)	5.0 (4.0–5.2)	<0.001*
Concentration post (sperm/mL)	6.5 (1.2–17.0)	4.0 (1.0–10.0)	0.09
Motility post (%)	70.0 (40.0–90.0)	90.0 (80.0–95.0)	<0.001*

Values are presented as median (interquartile range).

HBsAg: hepatitis B surface antigen.

*Statistical significance (p<0.05).

ies that highlighted how sperm quality may not be compromised in HBV patients [16,17].

Nevertheless, according to previous studies, it was confirmed a significant trend toward reduced sperm motility in HBV infected men. Indeed, other authors showed that HBsAg decreased sperm motility, generating the loss of sperm mitochondrial membrane potential [18]. On these bases, we can confirm previous theories that HBV also could produce chromosome aberrations, leading to hereditary defects in germinal cells [7]. This result could be one reason for lower total fertilization and CRs in couples with men partners being HBV-seropositive.

Of note, our primary outcome was to assess if the men viral infection could determine a real deterioration of the assisted reproductive outcomes. In this context, few previous studies focused on couples with only men infected, but the results remain controversial. Firstly, Zhao et al [19] demonstrated that couples with HBV husbands had similar FR, CR, and rate of good embryo quality compared with controls [20]. Subse-

quently, another study showed that HBV infected men had a higher risk of having a low FR during IVF, indicating that HBV had a deleterious effect on IVF outcomes [14]. Pirwany et al [12] observed lower implantation and PRs in HBV-positive individuals, compared to a healthy control group. The researchers recognized the limitation of the small sample size and they supposed that the extra precautions and handling techniques of the potential infective samples in the HBV group might have caused lower PRs linked to the controls. In contrast, Lam et al [13] described higher implantation and PRs in the HBV-seropositive group compared to those in the HBV-negative group. A more recent larger sample-size study observed that fertilization, implantation, and clinical PRs were significantly lower in HBV seropositive patients rather than seronegative [15].

Our results demonstrated that paternal HBV infection was related to significantly lower rates of total fertilization and cleavage. Nevertheless, no significant differences were found in the rates of implantation, clinical pregnancy, miscarriage and live birth between the HBsAg and control groups.

Therefore, one possible reason for the similar rates of pregnancy between the two groups was that other female factors, such as the endometrial receptivity, might play a key role. In this context, to reduce all possible confounding factors, we selected women with age <40 years, according to literature suggesting that in patients >or =42 years old a decline in clinical pregnancies, live births, as well as an increase of spontaneous abortions was found [21].

But yet, only one study considered the reproductive outcomes using the oocyte donation model, to reduce all possible bias related to women. Indeed, Bu et al [17], stated that couples with HBV seropositive husbands had similar seminal parameters, rates of fertilization, cleavage, implantation, and pregnancy, compared to their controls, suggesting that the infection has little impact on IVF outcomes.

Furthermore, we included in the study only men under the age of 45 years, according to previous studies

suggesting that IVF outcomes decrease with advancing paternal age [22].

Nevertheless, our work had some strengths and limitations. First of all, the retrospective nature of the study represents an important limitation; secondly, although previous studies involved very small study cohorts, the sample size of our study population remains small and lacks of statistical power. Moreover, the variations in oocyte quality and other factors from women's side were not well controlled, although patients of the two groups are matched for age, cause of infertility, and controlled ovarian stimulation protocol. Unfortunately, we were unable to consider only couples addressed to the ovum donation program, because of few patients discovered in our databank. Furthermore, prospective studies consisting of a large number of subjects are needed to get a clearer conclusion on this issue.

CONCLUSIONS

HBV infection proved to be able to affect fertilization and CRs in couples with HBsAg-positive men and negative women. However, clinical pregnancy outcomes, including implantation, pregnancy, miscarriage and live births rate were not influenced. In this setting, HBV infected men have the same chance to become father, compared to seronegative patients. Further prospective studies with larger sample size are needed to better understand the precise mechanisms of action of HBV infection on male fertility and reproductive outcomes.

Conflicts of Interest

The authors have nothing to disclose.

Author Contribution

Conceptualization: GC, MEC, RF. Data curation: GC, RF, RP, AC. Formal analysis: RP, RF, MS, FS, FR, EM, MT, LB, RC. Supervision: MEC, SS, MC, AN. Validation: SS, MC, AN, LC. Visualization: SS, MC, AN. Writing – original draft: GC, MEC. Writing – review & editing: AC, AN.

Data Sharing Statement

The data required to reproduce these findings cannot be

shared at this time due to technical and time limitations.

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