**Effects of hepatitis B virus S protein on human sperm function**

Xiao-Ling Zhou, Ping-Nan Sun, Tian-Hua Huang, Qing-Dong Xie, Xiang-Jin Kang, and Li-Min Liu

Research Center for Reproductive Medicine, Shantou University Medical College, Shantou 515041, People’s Republic of China

**Correspondence address. Tel: +86-754-88900845; Fax: +86-754-88900845; E-mail: thhuang@stu.edu.cn**

**Background:** Hepatitis B virus (HBV) has been determined to exist in semen and male germ cells from patients with chronic HBV infection, but no data are yet available on the impact of HBV S protein (HBs), the main component of HBV envelop protein, on the human reproductive system. The purpose of this article was to investigate the effect of HBs on human sperm function.

**Methods:** Sperm motility analyses, sperm penetration assays, mitochondrial membrane potential assays, immunolocalizations with confocal microscopy and flow cytometry analyses were performed.

**Results:** HBs reduced sperm motility in a dose- and time-dependent manner and caused the loss of sperm mitochondrial membrane potential. HBs–HBs monoclonal antibody (MAb) complex apparently aggravated such impairments. After 4 h incubation with HBs at concentrations of 25, 50, 100 μg/ml, the percentages of sperm motility a+b significantly decreased compared with the control (P<0.01). The fertilization rate and the fertilizing index in HBs-treated group were 40% and 0.57, respectively, which were significantly lower than 90% and 1.6, respectively, in the control (P<0.01). The asialoglycoprotein receptor (ASGP-R) and HBs were found to localize mainly on the post-acrosomal region. Both ASGP-R MAb and asialofoetuin, a high-affinity ligand of ASGP-R, inhibited the HBs-caused loss of sperm motility and mitochondrial membrane potential.

**Conclusions:** HBs had adverse effects on human sperm function, and ASGP-R may play a role in the uptake of HBs into sperm cells, as demonstrated by the competitive inhibition of ASGP-R MAb or asialofoetuin, resulting in diminished impairment caused by HBs.

**Key words:** asialoglycoprotein receptor / fertilizing ability / HBs protein / mitochondrial membrane potential / sperm motility

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**Introduction**

Hepatitis B is one of the major diseases of mankind and is a serious global public health problem. Of the 2 billion people who have been infected with the hepatitis B virus (HBV), more than 350 million have chronic (lifelong) infections. These chronically infected persons are at high risk of death from cirrhosis of the liver and liver cancer, diseases that kill about 1 million persons each year (WHO, 2000). Hepatitis B is also recognized as a sexually transmittable disease.

HBV is a double-stranded DNA virus belonging to the hepadnaviridae family and its proteins mainly consist of envelope proteins, the core antigen, a viral DNA polymerase and the X protein. The envelope proteins are composed of small envelope proteins (HBV S protein, HBs), middle envelope proteins and large envelope proteins (Alberti et al., 1990). The subviral particles of HBV, which predominantly comprise HBs, are produced in vast excess over HBV virions into the circulation, where concentrations reach 50–300 μg/ml (Ganem, 1996). HBs is also recognized as a pathogenic factor in some diseases. In fibrosing cholestatic hepatitis, immunohistochemistry and radioimmunoassays showed the accumulation of surface proteins in the hepatocytes (Foo et al., 2002). Furthermore, HBs may increase the risk of hepatocarcinogenesis (Zhou et al., 1999), and the recombinant HBs can interfere with or suppress the normal function of monocytes (Vanlandschoot et al., 2002; Cheng et al., 2005).

Although HBV replication is restricted to a more or less stringent host cell range, it is clear that viral integration is not restricted to any particular organ but occurs in many tissues including host somatic cells and spermatozoa (Naumova et al., 1985, 1986; Naumova and Kisselev, 1990; Xu, 1992; Otedo et al., 2003). It has now been demonstrated by many studies that HBV is able not only to pass through the blood–testis barrier and enter male germ cells but also to integrate into their genome (Scott et al., 1980; Hadchouel et al., 1985; Lang, 1993; Zhang et al., 1994; Zhao et al., 1998; Wang et al., 1999; Huang et al., 2002). After introduction into embryo by spermatozoa, they are able to replicate themselves and express viral proteins in the embryonic cells (Ali et al., 2005, 2006; Xiong et al., 2005). This process is known as father-to-infant vertical transmission of the infection that occurs in fertilization period. In contrast,
mother-to-infant vertical transmission of the infection occurs usually in the perinatal period. Vertical transmission of the infection is mainly seen in infants born from HBeAg-positive mothers with high levels of viral replication (Soderstrom et al., 2003).

Recent work in our laboratory has confirmed that sperm is a possible vector for vertical transmission of HBV (Huang et al., 2002; Ali et al., 2005, 2006). But still little is known about the influence of HBV infection on sperm functions, which is important for the fertility of HBV carriers. In 1985, Hadchouel et al. (1985) discovered HBV DNA in spermatozoa and proposed that HBV may be a cause of male infertility by damaging spermatozoa. Subsequently, it was reported that the frequency of sperm chromosome aberrations in an HBV-infected group (14.8%, 33/223) was significantly higher than that in the control (4.3%, 5/116) (Huang et al., 2003), and a reduced motility and a higher proportion of apoptotic and necrotic sperm in the patients with chronic HBV infection were observed (Moretti et al., 2008). The mechanisms of the HBV impacting on human sperm, however, remain unclear and intriguing. Therefore, this study addressed the aspects of the effects of HBs, the main component of HBV envelope proteins, on sperm functions including motility, mitochondrial membrane potential and fertilizing ability of sperm and the pathways closely involved in the process; these effects have not yet been documented in the previous studies.

### Materials and Methods

#### Ethical approval

Human sperm and mature female hamsters were used in sperm penetration assay. The animals were maintained under standard laboratory conditions (12 h light:12 h darkness cycle). The human sperm samples were collected from healthy male donors. All the protocols used in the present study were approved by the Ethical Review Committee of Shantou University Medical College and conformed to the National Institutes of Health guidelines for humane animal care and use in research and to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution’s human research committee.

#### Preparations of human spermatozoa

Semen samples, obtained from healthy men, were kept in a CO2 incubator (37°C, 50 ml/l CO2 in air) for 30 min to allow liquefaction. Motile spermatozoa were selected by the swim-up method as follows: in each test tube, 0.5 ml liquefied semen sample was layered gently under 2 ml of Biggers–Whittem–Whittingham (BWW) medium containing 0.3% bovine serum albumin (BSA) and incubated for 1 h at 37°C in a 5% CO2 incubator. The supernatant collected from three to four tubes was centrifuged at 100 g for 5 min, and the pellet of motile sperm was washed once and then resuspended in BWW medium with 0.3% BSA to a final concentration of 1 × 10^8 sperm/ml for the following studies.

#### Flow cytometry analysis

All flow cytometry (FCM) analyses were performed using a FACScan Flow Cytometer (BD Biosciences, San Diego, CA, USA) equipped with a single 488-nm argon laser. Forward and side scatters were gated on the major population of normal size cells. A minimum of 10,000 cells per sample were analyzed. The fluorescence signals of a monomer and an aggregate of 5,59,6,69-tetrachloro-19,3,39-tetraethylbenzimidazolocarbocyanine iodide (J-C1) were detected through the FL1 and FL2 channels, respectively. In the control experiments, the cells were pretreated with 100 nM valinomycin (Sigma-Aldrich, Shanghai, People’s Republic of China), a mitochondrial membrane potential-reducing K+ ionophore, at 37°C for 4 h (Cossarizza et al., 1994). Data were acquired in list mode, and the relative proportions of cells within different areas of the fluorescence profiles were quantified using the LYSYS II software program (Becton Dickinson). Results were expressed as the mean fluorescence intensity.

#### Sperm motility analysis

To investigate the influence of HBs on sperm motility, human spermatozoa (1 × 10^6/ml) were incubated in BWW medium with various concentrations of HBs (0, 25, 50, 100 μg/ml) (NCPC GencTech Biotechnology Co., Ltd, Hebei, People’s Republic of China) in a CO2 incubator (37°C, 50 ml/l CO2 in air). The sperm motility was assessed every hour using the WHO classification system (WHO, 1999), with the grades a (rapid progressive or linear motility), b (slow progressive or curvilinear motility), c (not progressive or in loco motility) and d (absent motility). In detail, ~20 μl sperm suspension was pipetted onto a clean, prewarmed sperm counter. A coverslip was lowered onto the sample, and the cell counter was examined on a microscope warm stage of phase contrast microscope with a ×40 objective by an experienced evaluator. At least five widely spaced fields were examined to provide an estimate of the percentage of motile cells. Each experiment was performed in triplicate and repeated at least three times using different donor samples. To investigate the influence of HBs–HBs monoclonal antibody (MAb) complex on sperm motility, human spermatozoa (1 × 10^6/ml) were incubated in BWW medium with 25 μg/ml HBs plus various concentrations of HBs MAb (0, 20, 40, 80 μg/ml) (Zhongshan Goldenbridge Biotechnology Co., Ltd, Beijing, People’s Republic of China). Sperm treated with 80 μg/ml HBs MAb only or left untreated were taken as controls. After 4 h incubation, the sperm motility was analyzed as described earlier.

#### Assessment of sperm mitochondrial membrane potential

The loss of mitochondrial membrane potential, an early marker for apoptosis, was quantified by FCM using the lipophilic cationic dye, JC-1 (Cossarizza et al., 1993). Energy released during oxidation reactions in the mitochondrial respiratory chain is stored as an electrochemical potential gradient consisting of a transmembrane electrical potential (ΔΨ), negative inside of ~180–200 mV. In normal cells, owing to the electrochemical potential gradient, JC-1 dye concentrates in the mitochondrial matrix, where it forms greenish-orange fluorescent aggregates (J-aggregates). Any event that dissipates the mitochondrial membrane potential prevents the accumulation of JC-1 dye in the mitochondria, and thus, the JC-1 dye is dispersed throughout the entire cell, leading to the color change of JC-1 changing reversibly from greenish-orange (J-aggregates) to green (J-monomers) as the mitochondrial membrane becomes disrupted (Smiley et al., 1991).

Assessment of mitochondrial membrane potential-reducing K+ ionophore, at 37°C, 50 ml/l CO2 in air) for 4 h. Subsequently, mitochondrial membrane potential was measured according to the manufacturer’s instruction using a JC-1 mitochondrial membrane potential assay kit (Invitrogen, CA, USA). Briefly, JC-1 was dissolved in dimethylsulfoxide (Sigma-Aldrich). An aliquot of 1 × 10^6 sperm cells was incubated in 0.5 ml liquefied semen sample was layered gently under 2 ml of Biggers–Whittem–Whittingham (BWW) medium containing 0.3% bovine serum albumin (BSA) and incubated for 1 h at 37°C in a 5% CO2 incubator. The sperm motility was assessed every hour using the WHO classification system (WHO, 1999), with the grades a (rapid progressive or linear motility), b (slow progressive or curvilinear motility), c (not progressive or in loco motility) and d (absent motility). In detail, ~20 μl sperm suspension was pipetted onto a clean, prewarmed sperm counter. A coverslip was lowered onto the sample, and the cell counter was examined on a microscope warm stage of phase contrast microscope with a ×40 objective by an experienced evaluator. At least five widely spaced fields were examined to provide an estimate of the percentage of motile cells. Each experiment was performed in triplicate and repeated at least three times using different donor samples. To investigate the influence of HBs–HBs monoclonal antibody (MAb) complex on sperm motility, human spermatozoa (1 × 10^6/ml) were incubated in BWW medium with 25 μg/ml HBs plus various concentrations of HBs MAb (0, 20, 40, 80 μg/ml) (Zhongshan Goldenbridge Biotechnology Co., Ltd, Beijing, People’s Republic of China). Sperm treated with 80 μg/ml HBs MAb only or left untreated were taken as controls. After 4 h incubation, the sperm motility was analyzed as described earlier.

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1 ml of PBS (Amresco Inc., Solon, OH, USA) containing 2 μM JC-1 at room temperature in the dark for 15 min. At the end of the incubation period, cells were washed and resuspended in 0.5 ml of PBS and immediately analyzed by FCM.

**Sperm penetration assay**

The mature female hamsters were induced to superovulate by intraperitoneal injection of 40 IU of pregnant mare serum gonadotrophin (Ningbo Hormone Product Co., Ltd, People’s Republic of China) on Day 1 of estrous cycle followed by administration of 40 IU human chorionic gonadotrophin (HCG, Ningbo Hormone product Co., Ltd) 72 h later. Superovulated oocytes were collected from the ampullar region of oviducts 17 h after HCG injection and freed from cumulus cells in 0.1% hyaluronidase (Sigma-Aldrich). Cumulus-free oocytes were washed twice in BWW medium with 0.3% BSA and treated with 0.1% trypsin (Sigma-Aldrich) to remove the zona pellucida and then washed twice immediately in BWW medium with 0.3% BSA.

The washed spermatozoa were suspended in 5 ml of 10 μM ionophore solution (Sigma-Aldrich) for 8 min to facilitate the capacitation and were washed twice and then divided into two groups including Group 1, which was treated with HBs protein (12.5 μg/ml) during the period of capacitation, and Group 2 as the control. The spermatozoa in both groups were suspended in BWW with 3.0% BSA and incubated in a CO2 incubator (37°C, 50 ml/l CO2 in air) for 4 h to allow capacitation. Insemination was undertaken with the sperm suspensions (10^6/ml). The oocytes were kept in the sperm suspension for 20–30 min and then transferred and incubated in BWW medium with 0.3% BSA under mineral oil (Sigma-Aldrich) for another h to ensure sperm penetration. After washing twice in ovum culture medium, the oocytes were examined for penetration under phase-contrast microscope after living oocytes were compressed between a slide and coverslip. An oocyte was recorded as being fertilized in the presence of a swollen sperm head or pronucleus with accompanying sperm tail (Yanagimachi et al., 1984). The fertilizing index is equal to the average number of swollen sperm heads and/or pronuclei with accompanying sperm tail per oocyte tested.

**Immunolocalization and confocal laser scanning microscopy**

After 4 h incubation with HBs, sperm samples were washed three times by PBS, fixed in methanol (−20°C) and mounted onto polylysine-pretreated glass slides. After rinsing twice with PBS, they were blocked in 5% normal goat serum/PBS for 30 min, rinsed three times and then incubated with anti-HBs mouse MAb at 4°C in a humid chamber for 16 h. The samples were rinsed and incubated with TRITC-conjugated goat anti-mouse IgG (Sigma-Aldrich) at room temperature for 40 min. For immunofluorescent localization of sperm asialoglycoprotein receptor (ASGP-R), the samples were incubated with FITC-conjugated anti-ASGP-R1 (BD7) mouse MAb (Santa Cruz Biotechnology, Inc., CA, USA) at room temperature for 1 h before mounting on coverslips with DABCO solution (Sigma-Aldrich). Observations were performed with confocal laser scanning microscopy (Nikon C1 Laser Scanning Confocal Microscope with three lasers: one argon laser, exciting at 488 nm, one HeNe laser, exciting at 546 nm, and one HeNe laser, exciting at 633 nm).

**Influence of ASGP-R MAb on sperm motility loss caused by HBs**

Sperm samples were divided into five aliquots. Four of them were incubated separately with 0, 1, 2, 3 μg/ml of ASGP-R MAb for 30 min and then incubated with 25 μg/ml HBs in a CO2 incubator (37°C, 50 ml/l CO2 in air) for 7 h, whereas one aliquot, the control, received no treatment before incubation. Sperm motility was assessed every hour starting from 5 h onwards using the WHO classification system (WHO, 1999).

**Influence of asialofetuin on sperm motility loss caused by HBs**

To explore the feasibility of an inhibitory effect of asialofetuin on sperm impairment caused by HBs, the spermatozoa were pretreated in BWW medium containing asialofetuin in various concentrations of 0, 0.5, 1 and 2 mg/ml at 37°C for 30 min and then incubated with 50 μg/ml of HBs in a humidified incubator (37°C, 50 ml/l CO2 in air) for 4 h. The sperm motility was analyzed as described previously.

**Statistical analysis**

Data were presented as mean values ± SD. SPSS 11.0 programs were used in the statistical analysis. After the homogeneity of variance test was carried out, data were analyzed by Independent-Samples t-Test. The fertilization rates and the fertilizing indexes were analyzed by χ^2 test. P-values <0.05 were considered to be significant.

**Results**

**Effect of HBs on sperm motility**

Human spermatozoa (1 × 10^6/ml) donated by a healthy donor were incubated in BWW medium with various concentrations of HBs (0, 25, 50, 100 μg/ml) in a CO2 incubator (37°C, 50 ml/l CO2 in air). The influence of HBs on sperm motility was evaluated by calculating the percentage of sperm motility a+b. The percentage of sperm motility c+d was eliminated due to the presence of apoptotic and necrotic spermatozoa. After 1 h incubation, the percentage of sperm motility a+b in the group that was treated with 100 μg/ml HBs decreased to 50 ± 4, which was significantly lower than that of the control group (98 ± 1) (P < 0.01), whereas there were no significant differences among the percentages of sperm motility a+b with 25 μg/ml HBs (96 ± 1) or 50 μg/ml HBs (96 ± 2) and the control (98 ± 1) group. After 4 h incubation, the sperm motility of all groups that were incubated with HBs, however, was decreased, although the control group still showed high motility. It was therefore observed that HBs reduced the sperm motility in a dose- and time-dependent manner (Fig. 1A).

Interestingly, the incubation of sperm with HBs and HBs MAb apparently accelerated the motility loss. The percentages of sperm motility a+b in the groups that were incubated with 25 μg/ml HBs plus HBs MAb (20, 40 and 80 μg/ml, separately) were 40 ± 6, 20 ± 3, 10 ± 3, respectively, which were significantly lower than 75 ± 4 motility in the sperm treated with HBs only (25 μg/ml) (P < 0.01). When compared with the normal control (the percentage of sperm motility a+b = 95 ± 2), the motility of sperm treated with HBs MAb only (the percentage of sperm motility a+b = 93 ± 2), even at the concentration of 80 μg/ml, was not affected (Fig. 1B).

**Loss of mitochondrial membrane potential caused by HBs**

The results showed that the sperm cells that lost mitochondrial membrane potential increased with increasing of concentrations of HBs, which was detected by a decrease in orange fluorescence emission. The percentages of sperm cells with depolarized mitochondria...
Localisation of ASGP-R and HBs on spermatozoa

In the present study, the localisation of ASGP-R and HBs on sperm was carried out by double staining with FITC-conjugated anti-ASGP-R1 (BD7) MAb (green) and TRITC-labeled HBs MAb (red) and laser scanning confocal microscope analysis. The results showed that the ASGP-R was localized in postacrosomal region, in the neck region and less intensely in the tail of sperm. ASGP-R and HBs were well presented mainly on the postacrosomal region of the sperm head (Fig. 3).

Inhibitive effect of ASGP-R MAb on sperm motility loss caused by HBs

The inhibitive effect of the binding of ASGP-R MAb on sperm motility loss caused by HBs was further investigated. The results (Fig. 4) showed that ASGP-R MAb, especially at lower concentrations (1 μg/ml), inhibited the adverse effect of HBs on sperm motility. After 5 h incubation, the percentage of sperm motility a+b in the group that was treated with HBs decreased to 50 ± 5, and motility in all groups that were treated with HBs plus ASGP-R MAb was ~80. Even after 7 h incubation, the percentage of motility a+b in the group that was treated with HBs plus 1 μg/ml ASGP-R MAb still remained at 70 ± 4, whereas the percentage of sperm motility a+b in the group that was treated with HBs only decreased to 1 ± 1. This demonstrated that HBs affected sperm motility through binding to ASGP-R. The binding of ASGP-R MAb to ASGP-R, by competitive inhibition, would protect the sperm from loss of motility induced by HBs. The protective effect, however, decreased with an increase of ASGP-R MAb concentration and with prolonging of the incubation time. After 7 h incubation, the percentages of sperm motility a+b in the groups that were treated with 25 μg/ml HBs plus 1, 2, 3 μg/ml ASGP-R MAb, respectively, became 70 ± 4, 60 ± 3 and 30 ± 4. During the 5, 6 and 7 h incubations, the percentages of sperm motility a+b in the groups that were treated with 25 μg/ml HBs plus 1 μg/ml ASGP-R MAb were 50 ± 5, 50 ± 4 and 70 ± 4, respectively.

Inhibitive effect of asialofoetuin on sperm impairment caused by HBs

Asialofoetuin, a high-affinity ligand of ASGP-R, also displayed a protective effect on sperm against HBs. It prevented the loss of sperm motility (Fig. 5). The percentages of sperm motility a+b in 0.5, 1 and 2 mg/ml asialofoetuin-pretreated groups were 61 ± 7, 66 ± 4 and 71 ± 4, respectively, which were significantly higher than that of the group treated with HBs only (20 ± 6) (P < 0.01). The assessment of mitochondrial transmembrane potential also indicated that the percentages of sperm with polarized mitochondria increased with the increase of asialofoetuin concentration, and the percentage of dead sperm decreased as the asialofoetuin concentration increased (data not shown). This indicated that asialofoetuin was adequately able to protect sperm from impairment caused by HBs.

Discussion

The study of the effects of viral proteins on sperm function and the understanding of the pathways involved are important for male reproductive health. In the present study, sperm motility, a widely used

Influence of HBs on human sperm fertilizing ability

Insemination was undertaken with the sperm suspensions from the HBs (12.5 μg/ml)-treated and control groups. Two indicators of sperm fertilizing ability, the fertilization rate and the fertilizing index in the HBs-treated group (40%, 0.57), were significantly lower than those in the control (90%, 1.6) (P < 0.01), which indicated that HBs reduce human sperm fertilizing ability (Table I).
fundamental indicator for male fertility, decreased greatly after incubation of sperm with 25–100 μg/ml HBs, and HBs reduce the sperm motility in a dose- and time-dependent manner. The incubation of sperm with HBs plus HBs MAb apparently accelerated motility loss because the percentages of sperm motility a+b in the groups incubated with HBs plus HBs MAb were significantly lower than that in the group treated with HBs only. The reason why HBs–HBs MAb complex accelerated the sperm motility loss is still unknown. Some studies have reported that the induced cellular product of HBs–HBs MAb exhibits a stronger cytotoxicity to T cells from mouse spleens than that of HBs alone (Zheng et al., 2001), and the antigen–antibody complexes deposited in the tissues have been found to damage tissues by triggering inflammation, which is recognized as the pathogenic mechanism underlying a variety of human diseases such as hepatitis B (polyarteritis nodosa), hepatitis C (cryoglobulinemia), HIV-related immune-complex nephropathy and so on. (Jancar and Sánchez Crespo, 2005). However, in the present study, the spermatozoa were in the simple medium and not in seminal plasma, and no inflammatory mediators could be present. Therefore, the mechanism by which HBs–HBs MAb complex accelerated the sperm motility loss might be different from the mechanism mentioned earlier.

The mitochondrial membrane potential represents the energetic state of mitochondria in a living cell and is often used to assess the activity of the mitochondrial respiratory chain, electrogenic transport systems and the activation of the mitochondrial permeability transition (Ly et al., 2003). Since the mitochondria of the sperm midpiece

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*P < 0.01, χ² test has been performed.

Figure 2 Effect of HBs on sperm mitochondrial membrane potential. JC-1 staining and FCM analysis of sperm cell were carried out following the different treatments. (A) 25 μg/ml HBs; (B) 50 μg/ml HBs; (C) 100 μg/ml HBs; (D) 25 μg/ml HBs+20 μg/ml HBs MAb; (E) the blank control; (F) valinomycin (positive control for depolarization). Abscissas, FL1-H values (green fluorescence, log scale); ordinates, FL2-H values (orange-red fluorescence, log scale).
generate energy to support motility, the state of sperm mitochondrial membrane potential is a useful indicator of functional impairment on the reproductive system (Gravance et al., 2001). There is a correlation between poor sperm mitochondrial function and diminished motility and reduced fertility (Marchetti et al., 2002; Piasecka and Kawiak, 2003; Wang et al., 2003). In the present study, human spermatozoa were incubated with the various concentrations of HBs, and HBs plus HBs MAb. It was detected that sperm mitochondrial membrane potential loss increased with the increase in HBs concentrations. The percentages of sperm cells with depolarized mitochondria in HBs plus HBs MAb-treated group was much higher than that in the group treated with HBs alone. Therefore, HBs caused the loss of sperm mitochondrial membrane potential, and the HBs–HBs MAb complex apparently aggravated this impairment.

Interspecific in vitro fertilization between human sperm and zona-free golden hamster ova established by Yanagimachi has been widely used in the area of reproductive biology (Yanagimachi et al., 1976), and was employed in the present study to investigate the influence of HBs on human sperm fertilizing ability. The fertilization rate and the fertilizing index of the HBs-treated spermatozoa were significantly lower than those in the control ($P < 0.01$), which indicated that HBs was able to reduce the human sperm fertilizing ability. This is probably because the HBs induced loss of sperm mitochondrial membrane potential and then diminished sperm motility leading to the decrease in sperm fertilizing ability.

ASGP-R is a glycoprotein receptor that recognizes ligands with terminal galactose and N-acetylgalactosamine residues and is hypothesized to function in the removal and recycling of desialylated glycoproteins from human serum (Stockert, 1995). ASGP-R was speculated to be involved in the pathogenesis of some viruses including HBV. It was reported that ASGP-R may play a role in the uptake of HBV into the hepatocytes (Treichel et al., 1994, 1997) and exist not only on the

**Figure 3** Confocal microscopy analysis showed the location of ASGP-R and HBs on the spermatozoa. Confocal laser microscopy images are representative of HBs-untreated (A) and HBs-treated (B) sperm cells by double-staining with FITC-conjugated anti-ASGP-R1 (8D7) MAb (green) and TRITC-labeled HBs MAb (red). (C) Enlarged photos from (B).
surface of hepatocytes but also on the surface of sperm cells (Abdullah and Kierszenbaum, 1989; Huber, 1992; Harvey et al., 2000). Previously, HBs-coated liposomes were found to be efficiently transported to the liver with nearly a 3-fold greater magnitude than the plain liposomes. And in vitro cell binding and uptake studies have revealed that HBs-coated liposomes are efficiently taken up by HepG2 cells by ASGP-R-mediated endocytosis (Khatri et al., 2005). The molecular pathway involved in the effects of HBs on sperm function was therefore further investigated in the present study. The results showed that the HBs were localized with ASGP-R mainly on the postacrosomal region of the sperm head. ASGP-R MAb, especially at a lower concentration, was able to inhibit the adverse effect of HBs on sperm motility. Even after 7 h incubation, the percentage of sperm motility a+b in the group that was treated with HBs plus ASGP-R MAb (1 μg/ml) still remained much higher than that in the group treated with HBs only. This demonstrated that HBs affected sperm motility through binding to ASGP-R, and the binding of ASGP-R MAb to ASGP-R, by competitive inhibition, was able to protect the sperm from motility loss induced by HBs. Before incubation with HBs, pretreatment with asialofetuin, a high-affinity and natural ligand of ASGP-R, also greatly reduced the sperm impairment caused by HBs. These results suggest that ASGP-R may play a role in the uptake of HBs into the sperm cell. Once the ASGP-R MAb or asialofetuin binds to the ASGP-R, the uptake of HBV into the spermatozoa would be affected owing to competitive inhibition, and the impairment from HBs would be diminished.

Another point worthy of mention here is that polysaccharides of HBs may play an important role in the binding of HBs to ASGP-R and in the pathogenic process involved. A potential N-glycosylation site at Asn-146, which is utilized partially for N-glycosylation, has been found in the domain of HBs (Heermann et al., 1984; Glebe and Urban, 2007). The lipopolysaccharides of other micro-organisms such as Neisseria gonorrhoeae and Chlamydia trachomatis are closely involved in sperm dysfunction (Harvey et al., 2000; Hosseinzadeh et al., 2001; Eley et al., 2005). This suggests that the molecular pathogenic mechanism by which HBs affect sperm function may be related to the polysaccharides of HBs.

In conclusion, the molecular pathogenic mechanism of HBs-induced dysfunction in human sperm needs to be further explored in the future. More attention should be paid to the reproductive health of the patients with HBV infection, especially with chronic infection, not only because HBV is able to be transmitted vertically to the offspring via the male germ line, but also because HBV may impair sperm function.

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