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Gender-specific alteration of steroid metabolism and its impact on viral replication in a mouse model of hepatitis B virus infection

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ABSTRACT

Hepatitis B virus (HBV) is a sex-specific pathogen that is more severe in males than in females. Sex disparities in HBV infection have been attributed to hormonal differences between males and females. However, whether HBV infection affects the metabolic signatures of steroid hormones and how these influences viral replication remains unclear. In this study, we investigated whether HBV infection alters steroid metabolism and its effects on HBV replication. Serum samples from male and female mice obtained after the hydrodynamic injection of replication-competent HBV plasmids were subjected to quantitative steroid profiling. Serum steroid levels in mice were analyzed using an *in vitro* metabolism assay with the mouse liver S9 fraction. The alteration of steroids by HBV infection was observed only in male mice, particularly with significant changes in androgens, whereas no significant hormonal changes were observed in female mice. Among the altered steroids, dehydroepiandrosterone (DHEA) levels increased the most in male mice after HBV infection. An *in vitro* metabolism assay revealed that androgen levels were significantly reduced in HBV-infected male mice. Furthermore, the genes involved in DHEA biosynthesis were significantly upregulated in HBV-infected male mice. Interestingly, reduced dihydrotestosterone in male mice significantly inhibits viral replication by suppressing HBV promoter activity, suggesting a viral strategy to overcome the antiviral effects of steroid hormones in males. Our data demonstrated that HBV infection can cause sex-specific changes in steroid metabolism.

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Introduction

The World Health Organization estimated that 254 million people (3.3%) worldwide were living with chronic hepatitis B virus (HBV) infection and that it caused approximately 1.1 million deaths, mostly from cirrhosis and hepatocellular carcinoma (primary liver cancer) in 2022 (WHO 2024).

Sex affects HBV replication and pathogenesis (Yu et al. 2001; Tian et al. 2012). Men are three to seven times more likely to carry HBV than women, and male HBV carriers have a higher risk of developing HCC (Yu et al. 2001; Chon et al. 2021). The mechanism underlying this sex disparity in HBV infection may be due to the differences in sex hormone levels between males and females. The risk of HCC is higher in male HBV carriers with higher serum androgen concentrations or androgen receptor (AR) alleles with higher transcriptional activity (Yu et al. 2000; Yu et al. 2001). In addition, sex hormones affect HBV-related liver diseases in a comprehensive manner by influencing the expression of sex hormone receptors or related transcription factors that promote or inhibit HBV replication (Chiu et al. 2007; Han et al. 2014). Androgens and their receptors are favorable for HBV replication (Wang et al. 2009; Tian et al. 2012), whereas estrogen receptors suppress HBV transcription (Wang et al. 2012).

Cholesterol is a crucial component of mammalian cell membranes and a precursor of steroid hormones. It plays important roles in membrane trafficking, transmembrane signaling, and cell proliferation (Goedeke and Fernández-Hernando 2012; Jung et al. 2022; Seo et al. 2023). Cholesterol is necessary for the HBV envelope and plays a critical role in HBV infectivity and viral particle secretion (Lin et al. 2003). Cholesterol depletion

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and cholesterol synthesis inhibitors such as inhibitors of 3-Hydroxy-3-Methylglutaryl-CoA Reductase or squalene cyclooxygenase impair the secretion of HBV viral or subviral particles in HBV-producing cell lines (Bremer et al. 2009). Over the last decade, the replication of human viral pathogens, including HBV (Iwamoto et al. 2014), are reportedly inhibited *in vitro* by oxysterols, physiological metabolites of cholesterol, whose action is linked to innate immunity (Lembo et al. 2016). Recently, a highly notable inverse correlation was found between the plasma levels of 25-hydroxycholesterol and 27-hydroxycholesterol and the active status of chronic hepatitis B (CHB) (Boglione et al. 2021). High level of 25-hydroxycholesterol (25HC) expression levels is associated with low HBV replication (Wei et al. 2022). Studies have shown that high NTCP expression induces an increase in bile acid transport to hepatocytes, which promotes HCV and HBV infection by the bile acid-mediated repression of some interferon-stimulated genes (Verrier et al. 2016; Eller et al. 2018). Also, elevated levels of intracellular bile acids activated FXRα, critical regulator of normal cholesterol metabolism, which enhanced HBV replication (Ramière et al. 2008; Zhao et al. 2018). In addition, FXRα silencing in HBV infected HepaRG cells decreased the pool size and transcriptional activity of viral covalently closed circular (ccc) DNA (Mouzannar et al. 2019). These findings indicate a strong association between HBV infection and the expression of steroid hormones, suggesting that HBV may affect their expression.

To this date, the roles of sex hormones in HBV and HBV-associated liver diseases have been relatively well studied, however, little is known about the alteration of sex hormones by HBV infection and how HBV infection regulates their expression. In this study, we investigated whether HBV infection could alter steroid metabolism and found that steroid levels were significantly altered only in male mice.

Materials and methods

Cell culture, transfection, and chemicals

Human hepatoma cell lines (HepG2 and Huh7) were obtained from the American Type Culture Collection (Manassas, VA, USA). All cell lines were cultured in DMEM (WELGENE, Gyeongsan-si, Korea) supplemented with 10% heat-inactivated FBS (Capricorn, USA) and 1% penicillin–streptomycin (Gibco, Grand Island, NY, USA), at 37°C in a 5% CO_2 humidified incubator. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for transient transfection according to the manufacturer's instructions.

Reference standards for all steroids analyzed (Ha et al. 2009) were obtained from Sigma (St. Louis, CA, USA) and Steraloids (Newport, RI, USA). Internal standards of 2,2,3,4,4,6,-d_6-dehydroepiandrosterone and 16,16,17-d_3-testosterone for androgens, 2,4,16,16-d_4-17β-estradiol for estrogens; 9,11,12,12-d_4-cortisol for corticosterones; and 2,2,4,6,6,17α,21,21,21-d_9-progesterone and 2,2,4,6,6,21,21,21-d_8-17α-hydroxyprogesterone for progestogens were purchased from C/D/N Isotopes (Pointe--Claire, Quebec, Canada). The trimethylsilylating agents, *N*-methyl-*N*-trifluorotrimethylsilyl acetamide (MSTFA), ammonium iodide (NH4I), and dithioerythritol (DTE) were purchased from Sigma-Aldrich.

Hydrodynamic injection of plasmid DNA in mice

A mouse model of acute HBV infection was established as previously described (Park et al. 2013) . Six-week-old male or female mice (n = 3–4 per group) were hydrodynamically injected with 25 μg of plasmid DNA, pHBV1.2 (wt) into the tail vein. Female mice were randomly selected regardless of their reproductive cycle because the primary goal of the present study was to compare sex differences against viral infection, not to investigate the role of each reproductive cycle during HBV viral infection. All procedures involving experimental animals were approved by the Animal Care Committee of the Konkuk University (KU15049). After 4 d, the mice were sacrificed and serum and liver tissues were harvested for gas chromatography-mass spectrometer (GC-MS) analysis, metabolic assay, Southern blotting, and real-time PCR.

HBsAg level in serum

Serum samples were collected one day after hydrodynamic injection. The serum samples were diluted to avoid signal saturation. The expression levels of secreted HBsAg were measured using an HBsAg detection enzyme-linked immunosorbent assay kit (Enzygnost HBsAg 5.0, Siemens, Erlangen, Germany) following the manufacturer's instructions. The optical density (OD) was measured at 450 nm using a spectrophotometer.

Southern blot analysis and HBV enhancer activity assay

The replication of HBV was detected using Southern blotting. Southern blotting was performed as previously described (Park et al. 2016; Dezhbord et al. 2024). The cells were transfected with plasmids harboring HBV and androgen receptors and treated with the indicated steroids. For the luciferase reporter assay, HepG2 cells

were grown to 40–50% confluence in 12-well plates. At 48 h post-transfection, the cells were harvested and lysed to measure luciferase activity using the Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

GC-MS analysis for steroid profiling

Blood samples were collected at 4 d after the hydrodynamic injection, and the serum was separated immediately and stored at −80°C until being used. The GC-MS-based serum steroid profiling was performed as described previously (Ha et al. 2009). Briefly, serum samples (100 µL) were spiked with 20 µL of internal standard mixture and diluted with 1 mL of 0.2 M phosphate buffer (pH 7.2). After loading the sample onto a preconditioned Oasis HLB cartridge, the SPE cartridge was washed twice with 1 mL of water and eluted with 2 mL of methanol and 2 mL of 90% methanol. The combined eluate was evaporated under a nitrogen stream at 40 °C. The sample was dissolved in 1 mL of 0.2 M acetate buffer (pH 5.2) and 100 µL of 0.2% ascorbic acid solution. Subsequently, the sample was extracted twice with 2.5 mL of methyl *tert*-butyl ether twice. The organic solvent was evaporated under a nitrogen stream at 40 °C and further dried in a vacuum desiccator with P_2O_5/KOH for at least 30 min. Finally, the dried residue was derivatized with 50 µL of MSTFA/NH_4I/DTE (500:4:2, v/w/w) at 60 °C for 20 min. Thereafter, 2 µL of the final mixture was injected into the GC-MS system.

Ex vivo *metabolic assay with liver S9 fraction*

Mouse livers were homogenized in five volumes of 20 mM potassium phosphate buffer containing 0.32 M sucrose and 1 mM DTT (pH 6.5). To remove the nuclei and other debris, the whole liver homogenate was centrifuged at 800 ×*g* for 10 min, and the resulting supernatant was subjected to further centrifugation at 9000 ×*g* for 30 min to yield the liver S9 fraction. The protein content of the final supernatant was assessed using Bradford assay.

Metabolic incubation was performed as previously described (Lee et al. 2016). The incubation mixture consisted of an NADPH-generating system (0.5 µmol $NADP^+$, 5 µmol glucose-6-phosphate, and 5 µmol $MgCl_2$), 20 or 50 µM DHEA, androstenediol (A-diol) or terstosterone, and 500 µg S9 fraction in a final volume of 0.5 mL in 40 mM potassium phosphate buffer (pH 6.5). Incubation was carried out at 37 °C for 1 h. All tested substrates were added under dry conditions and the incubation mixture was then added.

Table 1. Primers used for quantitative RT-PCR.

Gene	Forward primers	Reverse primers
Hsd3β	5′- GTG CCA GCC TTC ATC TTC T-3′	5′- AAG TGC CAC CAT TTT TCA GC-3′
Hsd17β2	5′-CAC GCT TCT CTG CGG ATG C-3′	5′-TGT GAA ACC CAG CTT GTC CA-3′
Hsd17β4	5′-AAA GCG GAA TCA GCC CAT GA-3′	5′-TTT GAC TGA CGC TCC CAC TC-3′
Gapdh	5′-AAC TTT GGC ATT GTG GAA GG-3′	5′-ACA CATT GGG GGT AGG AAC A-3′

Real-time PCR

Total RNA was isolated from hepatoma cells and mouse liver tissues using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was synthesized from total RNA (2 µg) using M-MLV reverse transcriptase (Intron Biotechnology, Seoul, Korea). Real-time PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and the primers used for RT–PCR (Table 1). cDNA amplification was conducted using ABI PRISM 7500 (Applied Biosystems). Relative quantification was analyzed by the comparative ΔΔCt relative to a calibrator. The results were presented as an n-fold difference relative to calibrator ($RQ = 2^{-\Delta\Delta Ct}$).

Data analysis

Data were obtained by at least three independent experiments and the values are presented as the mean ± standard deviation (SD). Based on individual steroid concentrations, the metabolic ratios of precursors to metabolites were calculated to indicate the corresponding enzyme activities. Differences between groups were tested for statistical significance using the Student's t-test or analysis of variance. Statistical significance was set at $P < 0.05$.

Results

Experimental design and overall scheme

To investigate whether HBV infection affects steroid metabolism, and whether altered steroids are associated with the replication and expression of viral proteins, we designed a scheme and performed experiments (Figure 1A). After expressing HBV in mice and confirming that the virus showed sex disparity, mouse sera were analyzed for steroid profiles using GC-MS. After isolating the microsomal S9 fractions from mouse liver tissue, *ex vivo* metabolism assays were performed to determine whether the altered steroids were caused by virus-induced differences in steroidogenesis. Finally, the effects of the differentially expressed steroids on the viral life cycle were analyzed using cell experiments.

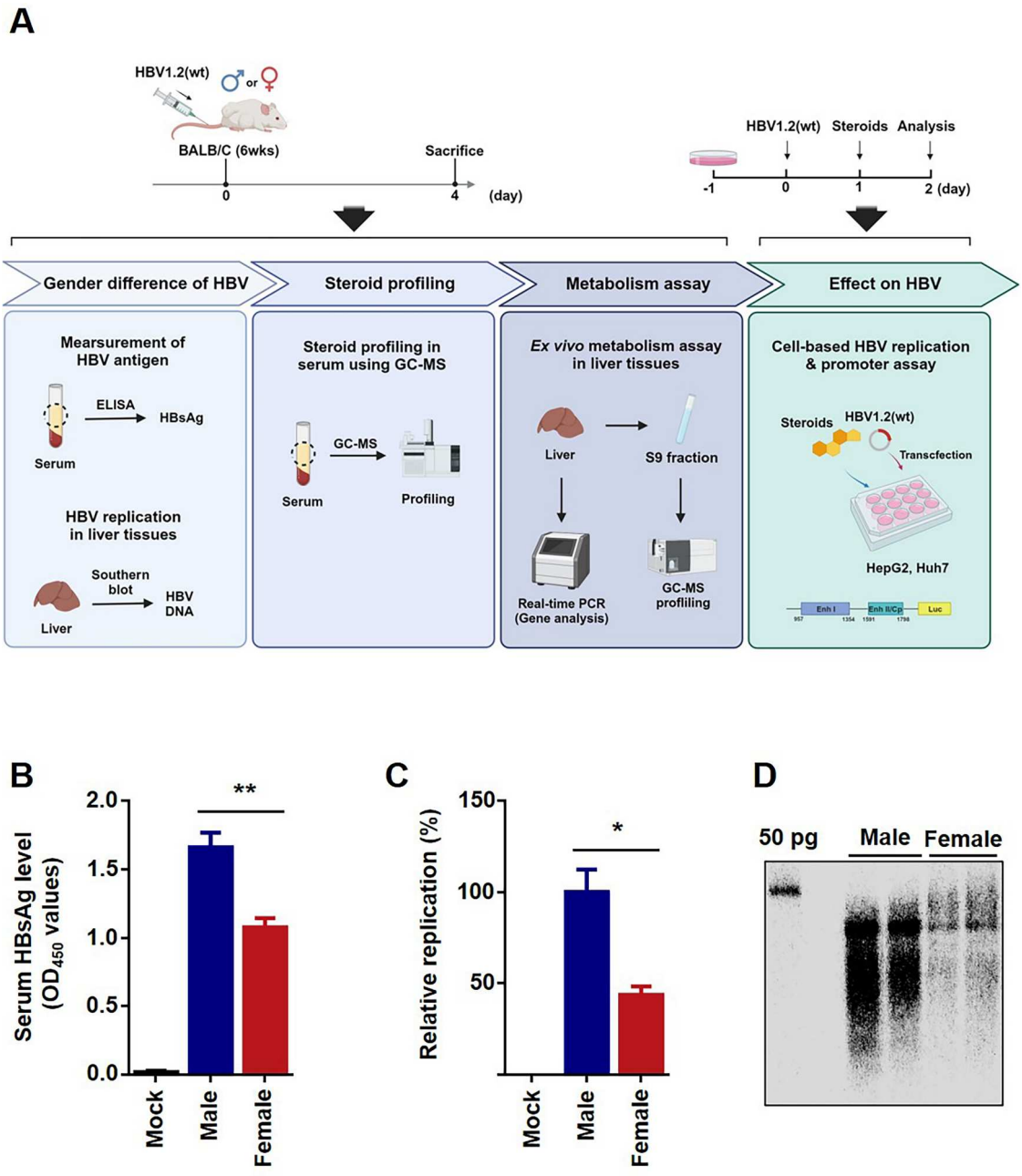


Figure 1. Experimental design and gender disparity of HBV infection in a mouse model of HBV. Overall scheme of the experimental procedure. After verifying the sex disparity in HBV infection in mice, changes in steroid hormones were evaluated using GC-MS, and steroid metabolism was analyzed in the liver tissue. The effects of altered steroid hormones on the life cycle of HBV were analyzed *in vitro* (A). HBsAg levels were measured in mouse serum 4 d after hydrodynamic injection of the HBV1.2 (wt) plasmid (B). Four days after hydrodynamic injection, mice were sacrificed, and liver tissues were used to analyze HBV replication in three or four mice per group (C). A representative image of Southern blot results (D). Values represent mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$.

Gender disparity of HBV infection

To investigate the effect of sex on HBV replication, we used a mouse model of HBV infection by hydrodynamically injecting the replication-competent HBV genome (HBV1.2(wt)), as described previously (Park et al. 2013; Dezhbord et al. 2024). We compared HBV replication and HBsAg levels in the liver tissue and serum of male and female HBV-infected mice on day four of infection. HBsAg expression and viral replication were higher in male than in females (Figure 1B, C). The level of HBV replication was more than twice as high in male mice than in female mice (Figure 1C, D). These results were

consistent with those of previous studies (Wang et al. 2009; Tian et al. 2012; Wang et al. 2012). Consequently, we conducted the following experiments using mouse tissue and serum samples, which were validated for their sensitivity to HBV in Figure 1.

HBV infection alters steroid metabolism only in male mice

Given the established relationship between HBV replication and the sex steroids androgens and estrogens, we hypothesized that HBV infection could potentially influence the metabolism of these steroids, thereby either benefiting or harming the virus or mice. To test this hypothesis, GC-MS analysis was conducted to investigate whether HBV alters serum steroid levels in infected male and female mice.

Steroid profiles were obtained from the serum samples (n = 3–4 per group) of male and female mice that showed sex disparities after HBV hydrodynamic injection. Significant changes in steroid levels caused by HBV infection were observed only in male mice (Table 2 and Figure 2). Among the steroids, three androgens and two corticoids were significantly altered in male mice, but no progestogens were monitored. In particular, the levels of androgens such as dihydrotestosterone (DHT), androsterone (An), and dehydroepiandrosterone (DHEA) were significantly altered (p <0.05); whereas those of other androgens, including androstenedione (A-dione), 5α-androstanedione (5α-dione) and testosterone (T), were not affected by HBV infection (Figure 2A). In addition, two corticoids, 11-dehydrocorticosterone (11-dehydroB) and allo-tetrahydro-11-deoxycorticosterone (allo-THS), were significantly altered by HBV infection ($p < 0.01$ both), whereas the other corticoids did not change in either male or female mice (Figure 2C). These findings imply that HBV infection may affect the metabolic pathways of steroids, specifically androgens and corticoids, exclusively in male mice, potentially affecting the host or HBV replication.

Ex vivo *steroid metabolic assay with mouse liver S9 fraction*

To examine whether the altered steroids in HBV infection resulted from changes in steroid biosynthesis, we performed an *ex vivo* metabolic assay using liver S9 fractions obtained from HBV-infected male and female mice. In particular, we focused on androgen metabolism pathways (Figure 3A) that were treated with DHEA, A-diol, and T.

Among quantitatively detected androgens in S9 fractions, most androgens were decreased in the liver of HBV infected male mice, while DHEA and its 16α-hydroxylated metabolite, 16α-OH-DHEA were increased after DHEA treatment (Figure 3B and C). In particular, 5β-androstane-3β,17β-diol (βββ-diol), A-dil, and etiocholanolone (Etio) levels were significantly decreased ($p < 0.05$). In contrast, no metabolic trends were found in the livers of female mice infected with HBV, except for 16α-OH-DHEA was significantly decreased. This might be due to the male-specific induction of

Table 2. Serum levels of steroid hormones in male and female mice of HBV infection model.

		Male				Female			
Steroids	Conc. (ng/mL)	Mock(n=3)	HBV(n=3)	Fold	*p* value	Mock(n=4)	HBV(n=4)	Fold	*p* value
Androgens	A-dione	ND	0.90(±0.78)		0.1167	ND	ND		
	DHT	0.60(±0.10)	trace 0.00(±0.00)	0.00	**0.0004**	ND	ND		
	An	0.79(±0.11)	0.56(±0.09)	0.71	**0.0486**	1.80(±0.64)	1.02(±0.23)	0.56	0.06073
	DHEA	2.24(±0.95)	15.00(±6.02)	6.71	**0.0222**	4.91(±4.62)	8.15(±6.78)	1.66	0.45967
	5α-dione	3.58(±3.21)	0.93(±0.19)	0.26	0.2274	ND	ND		
	T	3.05(±1.16)	1.53(±0.29)	0.50	0.0917	ND	ND		
	Etio	ND	ND			1.26(±0.22)	1.19(±0.58)	0.94	0.82857
Progestogens	Preg	ND	0.57(±0.50)		0.1205	ND	ND		
	Epi-*P*-one	3.02(±2.92)	0.87(±0.39)	0.29	0.2746	ND	ND		
	Iso-*P*-one	1.43(±0.26)	1.08(±0.39)	0.76	0.2705	ND	ND		
	20α-DHP	2.43(±0.91)	1.52(±0.23)	0.63	0.1709	5.91(±3.88)	5.25(±1.73)	0.89	0.7665
	P-one	15.61(±21.26)	6.69(±4.15)	0.43	0.5152	ND	ND		
	Allo-*P*-one	11.18(±3.55)	7.29(±1.65)	0.65	0.1604	ND	ND		
	5α-DHP	8.21(±1.76)	7.69(±0.74)	0.94	0.6646	ND	ND		
Corticoids	11-deoxyB	0.97(±0.16)	1.07(±0.05)	1.10	0.3716	ND	ND		
	allo-THB	1.54(±0.29)	1.41(±0.31)	0.92	0.6235	ND	ND		
	allo-THDOC	2.22(±0.26)	2.45(±0.21)	1.10	0.2938	ND	ND		
	allo-THS	2.36(±0.19)	1.95(±0.09)	0.83	**0.0272**	ND	ND		
	11-dehydroB	3.64(±0.49)	5.20(±0.31)	1.43	**0.0093**	ND	ND		
	allo-DHB	46.58(±16.22)	21.97(±4.14)	0.47	0.0636	13.78(±1.57)	16.79(±3.72)	1.22	0.18725
	B	385.34(±34.95)	466.90(±133.16)	1.21	0.3629	29.76(±12.50)	35.81(±3.21)	1.20	0.38466
	F	ND	ND			23.47(±7.82)	23.91(±1.36)	1.02	0.91482

ND: not detected.

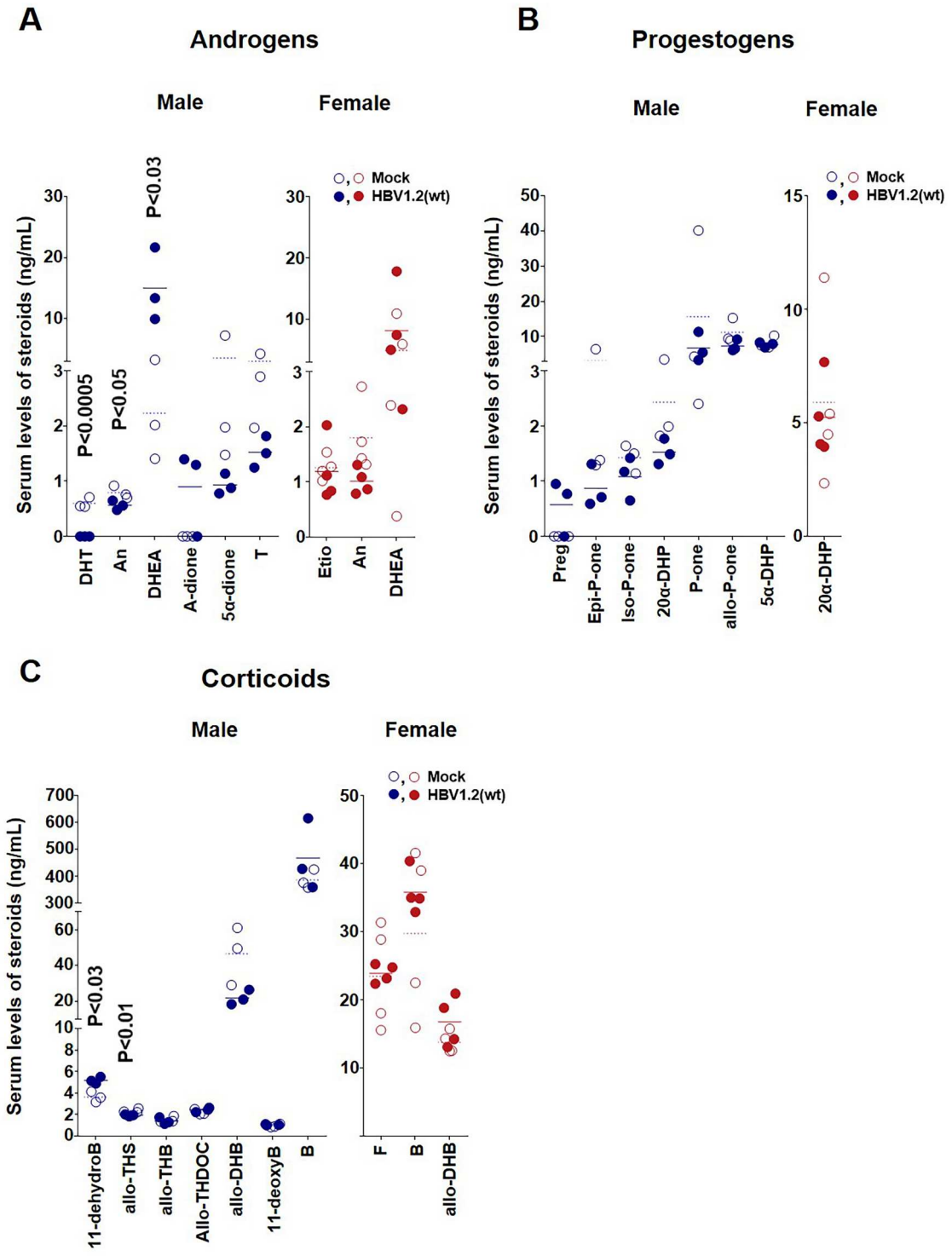


Figure 2. Comparative serum levels of steroid hormones between male and female mice in a HBV infection model. Four days after the hydrodynamic injection of HBV DNA, steroid hormone profiling was performed using GC-MS in mouse serum. Serum levels of androgens (A), progestogens (B), and corticoids (C) in male (blue) and female (red) mice (n = 3–4 each group). Empty circle, mock; closed circle, HBV infection. A-dione, Androstenedione; DHT, Dihydrotestosterone; An, Androsterone; DHEA, Dehydroepiandrosterone; 5α-dione, 5α-Androstanedione; T, Testosterone; Etio, Etiocholanolone; Preg, Pregnenolone; Epi-P-one, Epipregnanolone; Iso-P-one, Isopregnanolone; 20α-DHP, 20α-hydroprogesterone; P-one, Pregnanolone; Allo-P-one, Allopregnanolone; 5α-DHP, 5α-dihydroprogesterone; 11-deoxyB, 11-deoxycorticosterone; allo-THB, allo-tetrahydrocorticosterone; allo-THDOC, allo-tetrahydrodeoxycorticosterone; allo-THS, allo-tetrahydrodeoxycortisol; 11-dehydroB, 11-deoxycorticosterone; allo-DHB, Allodihydrocorticosterone; B, Corticosterone; F, Cortisol.

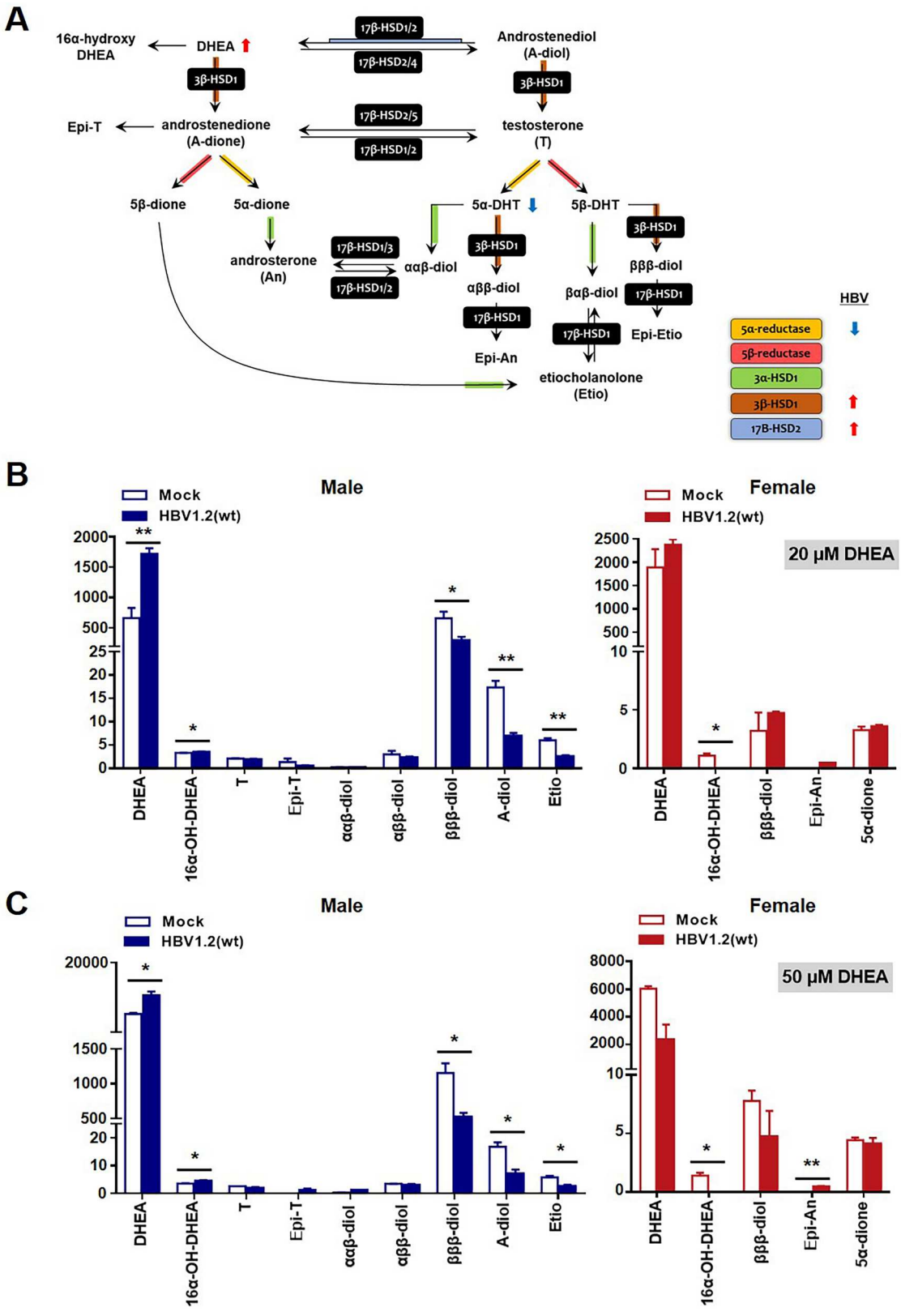


Figure 3. *Ex vitro* metabolic changes of androgens in mouse liver S9 fraction treated with testosterone precursors and testosterone. *Ex vitro* metabolic assay focusing on androgen metabolism was performed using pooled mouse liver S9 fractions. (A) Metabolic pathways of androgen. The effects of HBV infection on androgen levels and the corresponding genes in the metabolic pathway have been summarized. The upward red arrows indicate HBV-induced increases, whereas the downward blue arrows indicate decreases. Metabolic changes in androgens after treatment with DHEA (B and C), A-diol (D and E), and testosterone (F-H) were observed in male (blue) and female (red) mice. Values represent mean ± SD calculated from three independent experiments. *$p < 0.05$ and **$p < 0.01$.

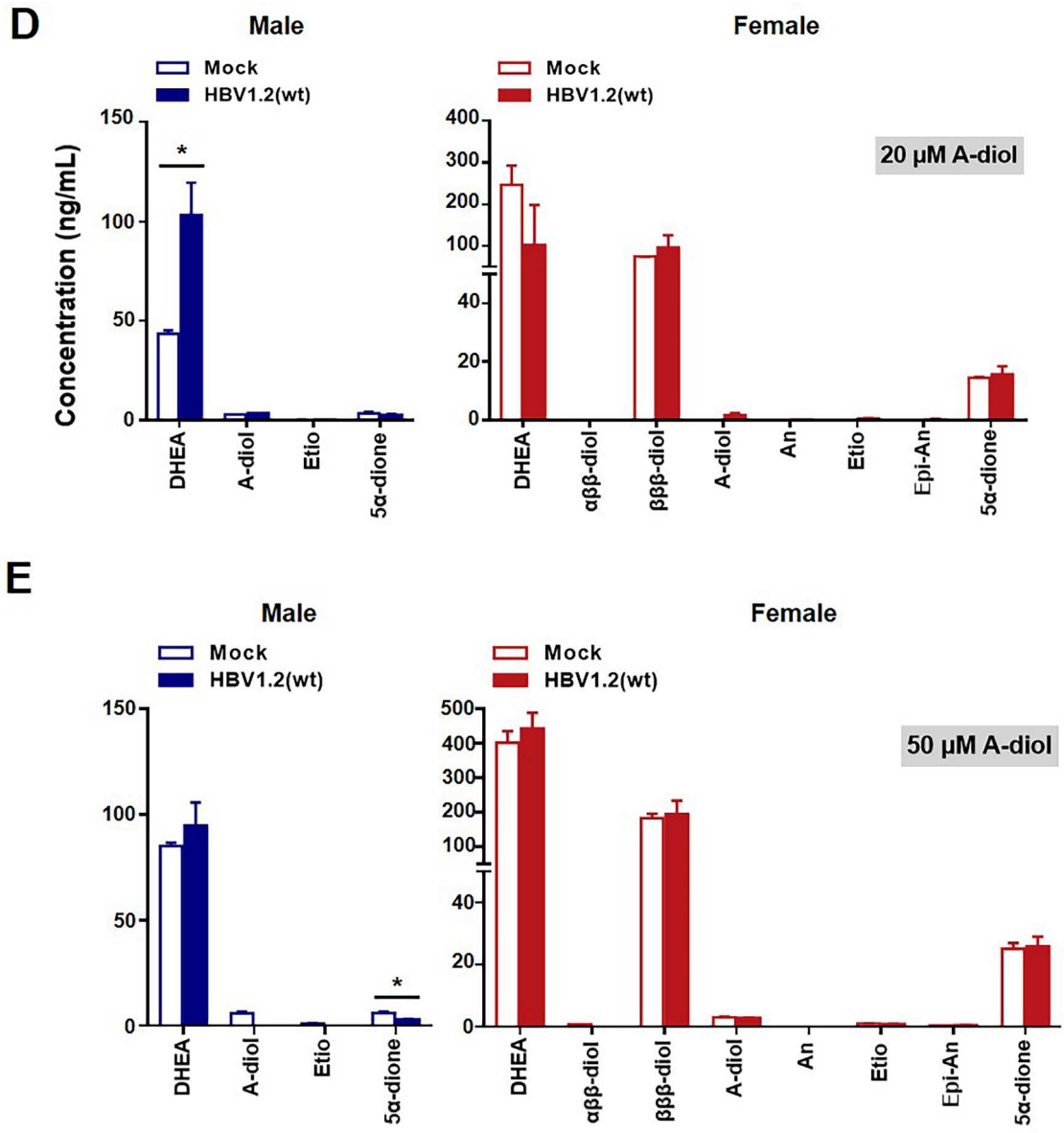
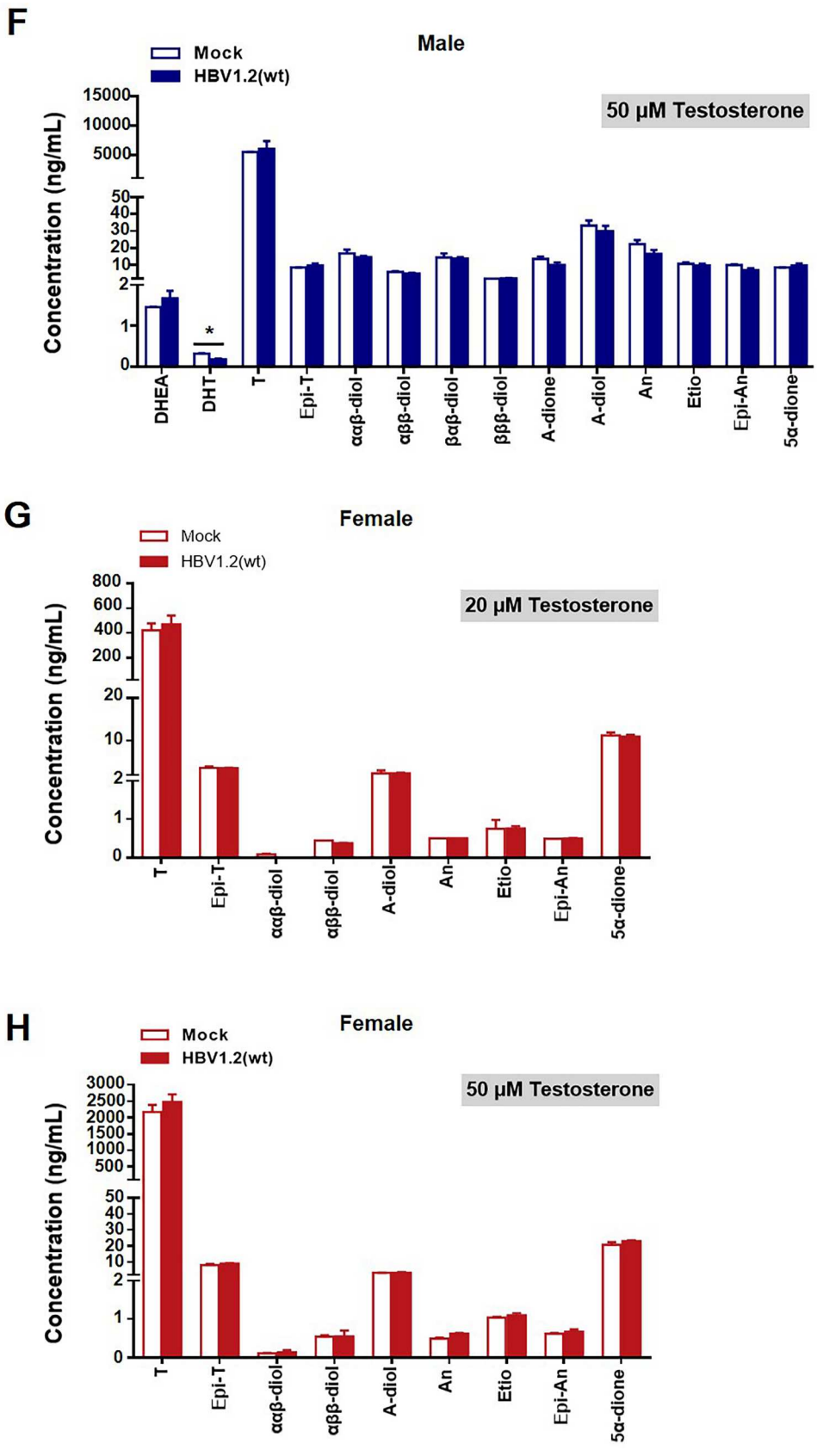


Figure 3. Continued

substrate-dependent Cyp2b9 (Hernandez et al. 2006). A precursor of T, A-diol, was also added to the mouse liver S9 fraction, and the accumulation of DHEA was observed ($p < 0.05$; Figure 3D and E), while 5α-dione was significantly decreased in male mice infected with HBV ($p < 0.05$; Figure 3E). In addition, we performed *in vitro* metabolic assay using T and found that only DHT levels were significantly decreased in HBV-infected male mice ($p < 0.05$; Figure 3F). However, no differences were observed in HBV-infected female mice (Figure 3G and H). Overall, these *ex vivo* data suggested that HBV infection could affect androgen metabolism, and the results were comparable to those in serum (Figure 2).

HBV infection regulates the genes involved in androgen metabolism

These results indicate that androgens are primarily altered by HBV infection in male mice. Therefore, we investigated whether the expression of genes involved in androgen metabolism was altered by HBV infection. In particular, we examined the major genes responsible for the biosynthesis and metabolism of DHT (Figure 3A), which showed the greatest changes due to HBV infection (Table 2 and Figure 2A). After HBV infection, the mRNA expression levels of 3β-Hsd type1 and 17β-Hsd type2 and 4 were determined in mouse liver tissues using real-time PCR. The expression of 3β-Hsd type1 ($p < 0.01$) and 17β Hsd type2 ($p <$



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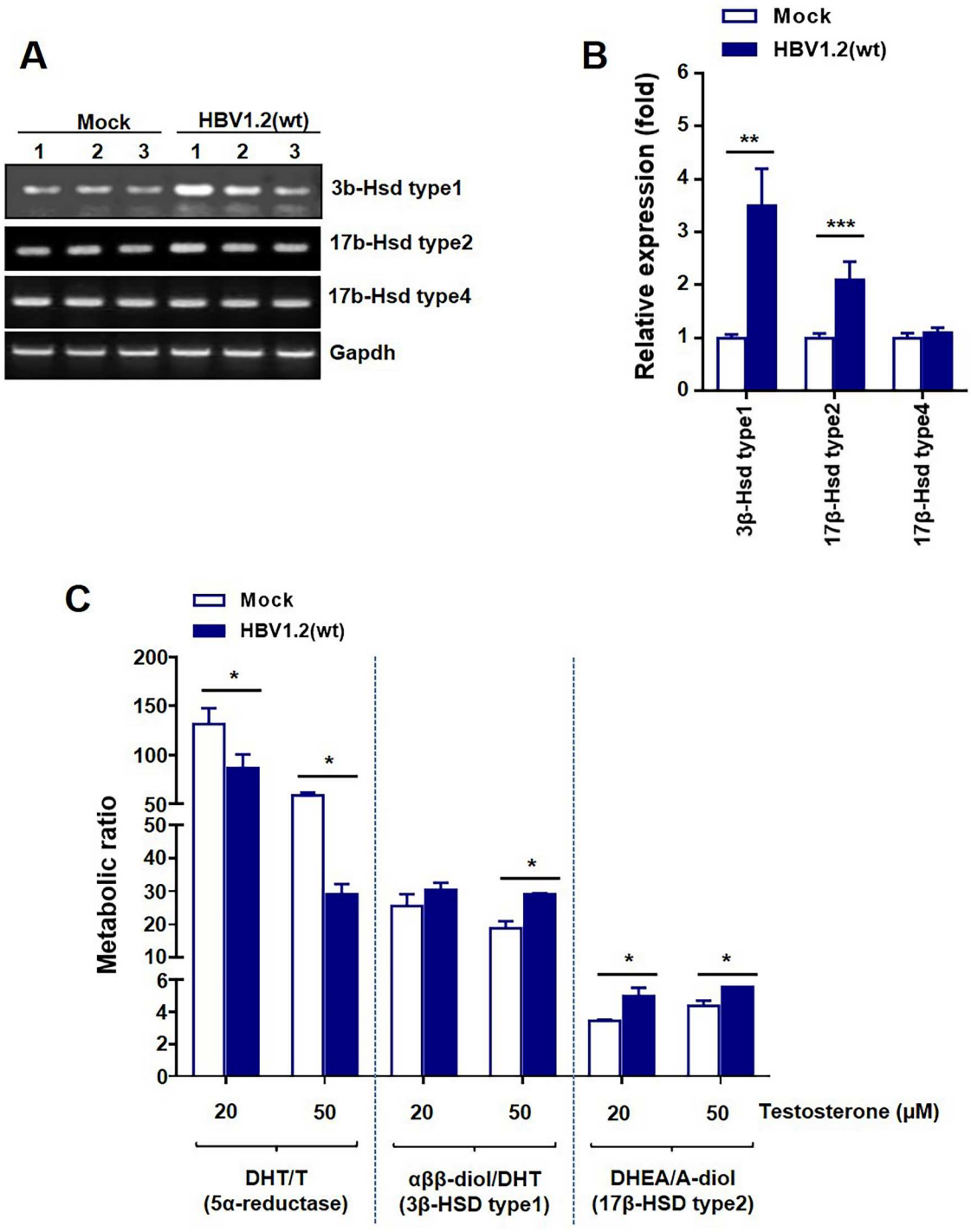

Figure 4. Effect of HBV infection on androgen metabolism in the mouse liver. Expression levels of the indicated genes in the liver tissues were analyzed using semi-quantitative RT-PCR (A) and quantitative real-time PCR (B). The metabolic ratios of precursors to metabolites were evaluated to determine the metabolic activity of the corresponding enzymes (C). Values represent mean ±SD calculated from three independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

0.001) was markedly increased by HBV infection (Figure 4A and B).

Based on the liver concentrations of individual androgens, metabolic ratios were measured to determine the activity of the enzymes involved in androgen metabolism. The metabolic ratio of DHT/T, corresponding for 5α–reductase activity, was decreased, whereas αββ-diol/DHT, responsible for 3β-Hsd type1 activity was increased (Figure 4C). The activity of the 17β-Hsd type2 enzyme, which metabolizes A-diol to DHEA (Mindnich et al. 2004; Moeller and Adamski 2006), was increased in the liver tissues

of HBV-infected mice compared to that in control liver tissues (Figure 4C). The changes in the activities of these enzymes were consistent with those of enzymes whose expression was altered by HBV infection (Figure 4A and B). Taken together, these data indicate that HBV infection alters the genes involved in androgen metabolic pathways and, in turn, alters their activity. These changes likely contributed to the altered steroid profiles in HBV-infected male mice, as shown in Figure 2A (Figure 3A).

Effect of androgens altered by HBV on viral replication

To investigate the effect of androgens on the viral replication altered by HBV infection (Figure 5A), we assessed the level of viral replication using Southern blot analysis after androgen treatment (Figure 5B). Of the treated androgens, only DHT and 5α-dione were significantly altered during viral replication (Figure 5B and C). Interestingly, HBV replication was markedly inhibited by DHT treatment in both Huh7 and HepG2 cells (Figure 5B and C). DHT inhibited HBV replication in a dose-dependent manner (Figure 5D). Serum levels of DHT in HBV-infected male mice were also significantly reduced ($p = 0.0004$; Table 2), suggesting that it may have a beneficial role against the virus. In contrast, 5α-dione significantly increased HBV replication ($p < 0.01$) (Figure 5B and C) and enhanced HBV replication in a dose-dependent manner (Figure 5E). However, the change in the serum levels in HBV-infected male mice was not statistically significant (Figure 5A).

To elucidate how DHT inhibits HBV replication, we used a reporter luciferase plasmid containing key HBV enhancer and promoter regions (Enh I and Enh II/Cp) (Figure 5F), as described in our previous report (Kim et al. 2018). DHT significantly inhibited the activity of the HBV enhancer ($p < 0.01$), whereas the other androgens, including 5α-dione, did not reduce promoter activity. This finding implies that DHT suppresses HBV transcription, whereas 5α-dione promotes viral replication via a distinct mechanism.

Discussion

Hepatitis B virus (HBV) is an important pathogen that chronically infects males more frequently than females. This characteristic of HBV infection has been attributed to the sex hormones in the host. In this study, we investigated whether HBV infection altered steroid profiles. We observed significant changes in androgen levels in male mice infected with HBV, whereas no significant HBV-induced differences were observed in female mice (Figure 2). *Ex vivo* metabolic analysis of HBV-infected male mice further confirmed that the virus modulated steroid metabolism (Figure 3). We also found that several genes involved in androgen conversion were significantly upregulated in male HBV-infected mice. Overall changes in the genes and steroid profiles involved in steroid metabolism following HBV infection are shown in Figure 3A. Importantly, DHT, an androgen altered by HBV infection, affected HBV replication in Huh7 and HepG2 hepatoma cell lines (Figure 5).

The biogenesis networks involved in androgen metabolic pathways (Figure 3A) are extremely complex. Therefore, explaining the exact mechanism of the HBV-induced changes in steroid profiles in male mice is difficult as shown in Figure 2A based on only a few gene changes and their activity assays (Figure 4) and *ex vivo* steroid metabolism assay with the mouse liver S9 fraction (Figure 3). However, this study evidently indicates that HBV infection can alter the metabolic pathways of androgens and thus alter androgen levels in the infected serum.

Previously, 5α-reductase, which converts T to DHT, was reported to have a central role in mammalian male physiology. Steroid profiling analysis showed that level of DHT infection significantly decreased the DHT levels in male mice (Figure 2A and Table 2). Thus, we investigated whether HBV infection alters the enzymatic activity involved in the biosynthesis of DHT and its metabolism in the liver. Data showed that the 5α-reductase activity was markedly reduced in the liver tissues of HBV infected male mice after T treatment (Figure 4C), resulting reduced levels of serum DHT in mice. HBV infection can increase the incidence and risk of male infertility (Su et al. 2014), and decreased serum levels of DHT controlled by 5α-reductase inhibitors have negative effects on libido and erectile function (Chiba et al. 2011; Drobnis and Nangia 2017). Further studies on this topic will improve our understanding of HBV infection and male reproduction.

Acute-on-chronic liver failure (ACLF) is a complex syndrome characterized by acute and severe liver injury in the context of pre-existing chronic liver disease (Bernal et al. 2015). In Asian populations, the major underlying cause of ACLF is chronic HBV infection (Sarin et al. 2019). HBV-ACLF is associated with a high short-term mortality rate ranging from 58% to 74% (Wu et al. 2018). Recently, the baseline T levels in the HBV-ACLF group were reported to be significantly lower than those in the non-ACLF group (Sun et al. 2021). Our data showed that male HBV-infected mice had lower T cell levels (Figure 2A and Table 2). This may be related to the lower T levels in the HBV-ACLF group; the

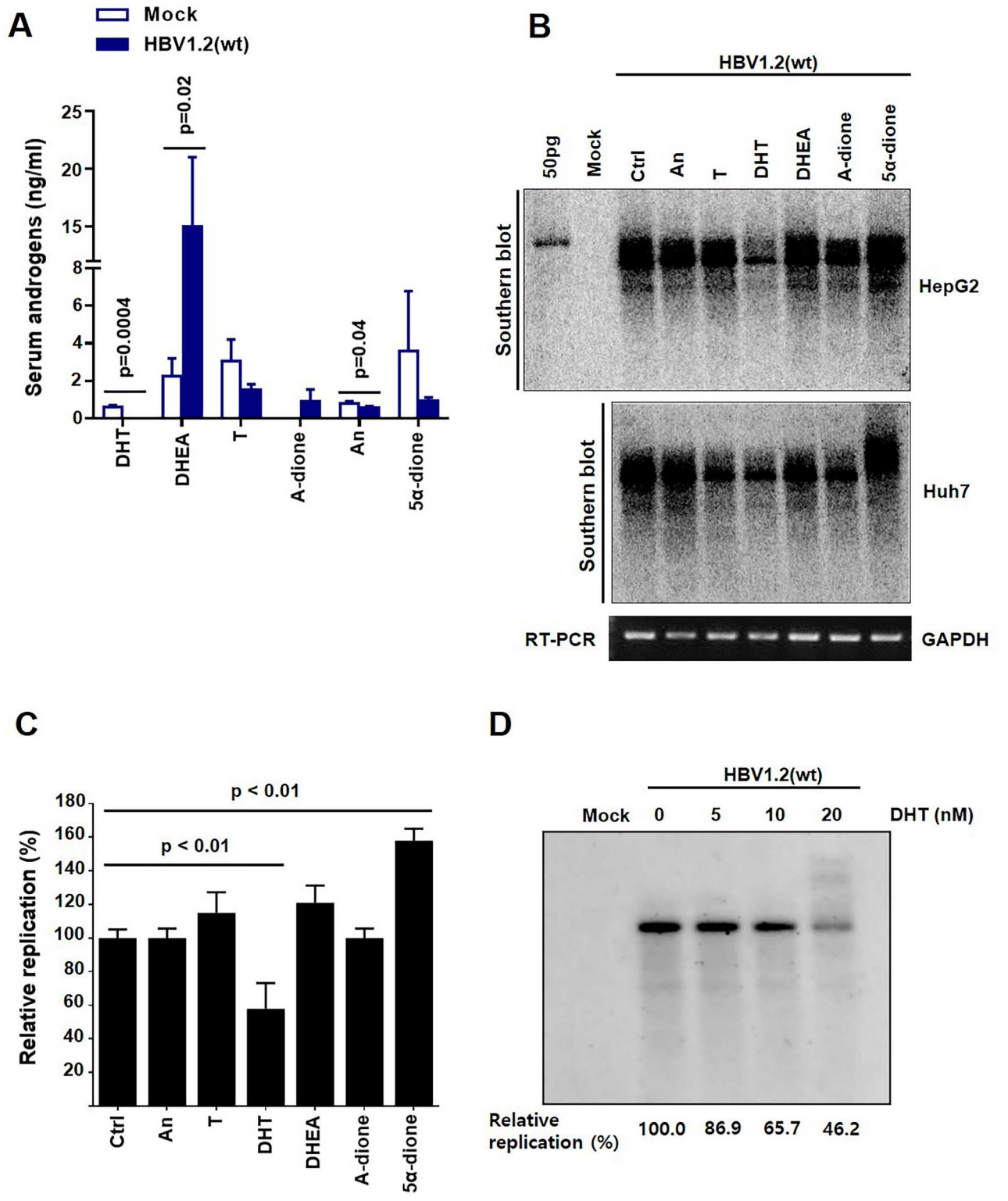

Figure 5. Effect of altered androgens on HBV replication and enhancer/promoter activity. (A) Altered androgen levels in serum after HBV infection in male mice. (B-E) Effects of altered androgens on HBV replication. Huh7 and HepG2 cells were transfected with HBV1.2(+) and treated with the indicated steroids (20 nM each). HBV replication was determined using Southern blot analysis. HBV DNA levels were quantified using Multi-Gauge V3.0, and plotted (C). Dose-dependent effect of DHT (D) and 5α-dione (E) on HBV replication in HepG2 cells. (F) Effect of altered androgen levels on HBV enhancer/promoter activity. Activity of the HBV enhancer/promoter was analyzed using a luciferase assay. Values represent mean ± SD calculated from three independent experiments.

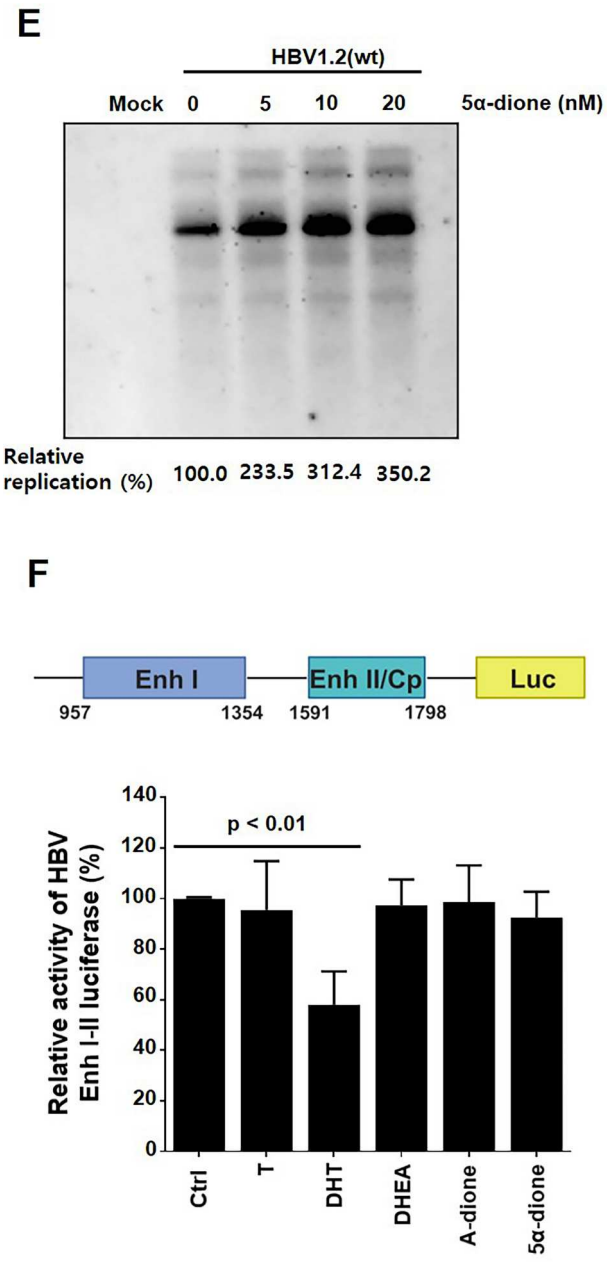

Figure 5. Continued

correlation between lower T levels and clinical outcomes requires further investigation.

HBV X protein (HBx) enhances androgen receptor (AR)-responsive gene expression in an androgen concentration-dependent manner (Chiu et al. 2007). Among the steroids altered by HBV infection, DHT and 5α-dione caused significant changes in viral replication. The virus-enhancing effect of 5α-dione was not surprising, as androgens and their receptors are favorable for HBV replication (Wang et al. 2009; Tian et al. 2012). However, DHT significantly inhibited HBV replication in both Huh7 and HepG2 cells (Figure 5B-C). Serum DHT levels were significantly reduced in HBV-infected male mice (Table 2 and Figure 5A). This may be a viral compensatory mechanism in male mice, in which HBV inhibits the production of antiviral DHT, creating a favorable environment for HBV replication. This is supported by the finding that DHT directly inhibits enhancer and promoter activities (Figure 5D), which regulate the overall transcription of the HBV cccDNA minichromosome. However, the regulation of DHT expression by HBV remains to be elucidated in further studies.

Although this study mainly investigated the effects of androgens altered by HBV infection on HBV replication, long-term changes in these hormones due to chronic hepatitis B may be more likely to affect some diseases in the host than the HBV life cycle. For example, the production rate of androgens is associated with age-related disorders such as insulin resistance, cardiovascular disease, and osteoporosis (Papadopoulou-Marketou et al. 2000); therefore, further studies on chronic hepatitis B, levels of DHEA, T, and DHT, and their metabolic association with these diseases are warranted (Moulana et al. 2011).

Female rodents such as mice and rats have four sequential phases in their reproductive cycles: proestrus, estrus, metestrus, and diestrus (Spornitz et al. 1994; Ajayi and Akhigbe 2020; Zhou et al. 2022). Each stage has different hormone levels and responsiveness. This study primarily aimed to compare sex differences in response to HBV infection. Therefore, we randomly selected female mice regardless of their reproductive cycle. However, the response of females to viral infections may vary across different reproductive cycles, which could contribute to the relatively small variation observed in females investigated in this study. For example, during the diestrus stage, hormonal responsiveness might be similar to that in males, whereas during the estrus cycle, a lack of response or an opposite response compared to males could be expected. This random mixture could have potentially led to the results observed in this study. Hence, future studies should compare the responsiveness to viral infection across different reproductive cycles to identify these differences. Additionally, translational research is needed to understand the clinical significance of these findings in relation to the human menstrual cycle and pregnancy.

In conclusion, we found that HBV infection affects steroid profiles, as assessed by GC-MS, in a male HBV mouse model, implying that HBV may alter androgen metabolism in a sex-specific manner. Furthermore, we discovered that HBV infection decreased DHT levels, and that DHT inhibited viral transcription and replication. This finding suggests an important link between HBV infection and androgen metabolism in male hosts.

Authors' contributions

Study conception and design: Eun-Sook Park and Kyun-Hwan Kim; Acquisition of data: Eun-Sook Park, Sung Hyun Ahn, Ah Ram Lee, Donghyo Lee, and Ju-Yeon Moon; Analysis and interpretation of data: Eun-Sook Park, Ju-Yeon Moon, Man Ho Choi, and Kyun-Hwan Kim; Material support: Man Ho Choi; Obtained funding: Eun-Sook Park and Kyun-Hwan Kim; Drafted the manuscript: Eun-Sook Park, Man Ho Choi, and Kyun-Hwan Kim.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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