EFFECT OF ENTECAVIR AND ATORVASTATIN IN A DUCK MODEL OF HEPATITIS B COMBINED WITH NONALCOHOLIC FATTY LIVER DISEASE

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ABSTRACT

Introduction: *To evaluate the effect of entecavir and atorvastatin in a duck model of Hepatitis B virus (HBV) combined with nonalcoholic fatty liver disease (NAFLD).*

Materials and methods: *A total of 75 Guangdong sheldrakes with congenital infection of duck hepatitis B virus (DHBV) were included. Among which, 15 ducks were fed with normal fodder (sham group). Other 60 ducks were fed with a high-fat and high-sucrose diet which consists of 83% duck fodder, 10% lard, 5% sugar, and 2% cholesterol for 3 weeks to build the duck model of NAFLD, then they were divided into 4 groups to receive different therapies, 1) entecavir and atorvastatin group (E&A group); 2) entecavir group (E group); 3) atorvastatin group (A group) and 4) control group. Each group had 15 ducks. The levels of liver function (alanine aminotransferase [ALT], aspartate aminotransferase [AST], glutamyl transpeptidase [GGT], and alkaline phosphatase [ALP]), lipid metabolism (triglyceride [TG], cholesterol [TC], high-density lipoprotein cholesterin [HDL-C], low-density lipoprotein cholesterin [LDL-C] and glucose [Glu]), and DHBV Deoxyribonucleic Acid (DNA) in serum were determined at baseline, day 7, day 14 and day 21, respectively. Intrahepatic DHBV DNA levels were measured on day 21.*

Results: *It is shown that all parameters of liver function and lipid metabolism in ducks with NAFLD were significantly higher compared with those in the sham group, showing that the model was built successfully. Repeated regression analyses showed that all liver function and lipid metabolism parameters in the E&A group were significantly lower compared with those in the E group, A group, and control group. In addition, both serum and intrahepatic DHBV DNA levels were significantly lower in the E&A group compared with those in other groups.*

Conclusion: *Combination of entecavir and atorvastatin improves hepatic steatosis and increases antiviral responses in the duck model of HBV and NAFLD.*

Keywords: *Entecavir, atorvastatin, hepatitis B virus, nonalcoholic fatty liver disease, duck.*

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Introduction

Hepatitis B virus (HBV) infects more than 350 million people worldwide, which is a common cause of liver diseases and liver cancer and leads to 1 million deaths each year[1]. Although anti-HBV vaccines are effective and available, the number of patients suffering from chronic hepatitis does not decrease substantially, especially in low- and middle-income countries[2]. Nonalcoholic fatty liver disease (NAFLD) is a disorder characterized by abnormal accumulation of fat in the liver of a patient who drinks little or no alcohol[3]. Accompanied by the growing number of patients with obesity and hyperlipemia, the prevalence of NAFLD has been increasing globally in the past years[4]. It is estimated that 25% of populations have NAFLD[5], among which 15%-20% may progress to nonalcoholic steatohepatitis (NASH), and potentially progress to more serious disease states such as cirrhosis and hepatocellular carcinoma[6]. Along with the increasing number of patients with NAFLD, concurrent infection of

NAFLD and HBV has also increased. It is reported that NAFLD and HBV synergistically aggravate liver injury through oxidative damage, thereby promoting hepatic fibrosis and hepatocellular carcinoma[2, 7, 8]. Therefore, exploring novel treatments is an urgent need. Entecavir is a guanosine nucleoside analog used for treating HBV infection. It is now recommended as a first choice for HBV patients in most international guidelines[9]. Thus far, there is no proving strategy for NAFLD, current investigations mainly concentrate on treating constituents of metabolic syndrome. It is widely recognized that statins could reduce low-density lipoprotein (LDL) levels by interfering with cholesterol production in the liver, thereby alleviating dyslipidemia, a hallmark of metabolic syndrome[10]. The present study aims to evaluate the effect of entecavir and atorvastatin in a duck model of HBV combined with NAFLD.

Materials and methods

Animals

A total of 75 one-day-old Guangdong sheldrakes with congenital infection of duck hepatitis B virus (DHBV) were purchased from a commercial hatchery and held in the animal house facilities at our hospital. All animal handling procedures were approved by the animal ethics committee of our hospital and followed the Chinese guideline for ethical review of animal welfare.

Treatments

Fifteen ducks were fed with normal fodder (sham group). When other 60 ducks were 3-months old, they were fed with a high-fat and high-sucrose diet which consists of 83% duck fodder, 10% lard, 5% sugar, and 2% cholesterol for 3 weeks to build the duck model of NAFLD. Then they were divided into 4 groups to receive different therapies.

- Entecavir and Atorvastatin group (E&A group), 15 ducks were orally administrated at a dose of 1 mg/kg/day of entecavir and 3 mg/kg/day of atorvastatin;
- Entecavir group (E group), 15 ducks were orally administrated at a dose of 1 mg/kg/day of entecavir;
- Atorvastatin group (A group), 15 ducks were orally administrated at a dose of 3 mg/kg/day of atorvastatin;
- Control group, 15 ducks received the distilled water as a placebo.

All ducks were weighted weekly for calculating the doses and treatments were administrated for 21 days. On day 21, all ducks were sacrificed, and their livers were removed and stored frozen for subsequent analysis of viral Deoxyribonucleic Acid (DNA).

Liver function and lipid metabolism evaluation

Blood samples were collected from the jugular vein at baseline (D0), day 7 (D7), day 14 (D14), and day 21 (D21), and serum was collected following incubation of the blood samples at 37°C for 90 min and then stored at -20°C. Serum samples were tested on the day of collection for levels of liver enzymes and lipid metabolism. Liver enzymes include alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP). Lipid metabolism parameters were triglyceride (TG), cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low- density lipoprotein cholesterol (LDL-C), and glucose (Glu).

DHBV DNA in serum and liver

Total DHBV DNA levels in serum and liver DNA extracts were assessed by the modified slot blot DNA assay. Covalently closed circular DNA (cccDNA) was purified from liver homogenates as described previously[11] and was analyzed by electrophoresis through 1% agarose gels followed by blotting onto nylon membranes (Hybond N; Amersham Pharmacia Biotech, Piscataway, N.J.). Hybridization was performed as described previously[11] with a HiPrime Random Primer kit (Roche Diagnostics Corporation, Shanghai, China) for radiolabeling.

Statistics analysis

Continuous variables are expressed as mean±standard deviation (SD) and compared by One-Way Analysis of Variance (ANOVA), followed by post-hoc analysis. Repeated measures ANOVA was performed to assess the differences in liver enzymes, lipid metabolism parameters, and DHBV DNA levels among groups during various time points. All tests were 2-sided and a P value of less than 0.05 was considered significant. All statistical analyses were performed with the SPSS statistical software program package (IBM SPSS Statistics for Windows, Version 20.0. IBM Corp. Released 2011. Armonk, NY, USA).

Results

Table 1 illustrates the baseline lipids and liver enzymes detected in five groups. It is shown that the

baseline DHBV in serum and HDL-C levels were similar among five groups (P=0.81) at baseline.

	Sham group	Control group	E group	A group	E&A group	*P*
DHBV in sera (lg copies/ml)	8.4±0.7	8.3±0.5	8.4±0.6	8.7±0.8	8.5±0.6	0.81
ALT (U/L)	36.3±4.7	200.1±18.4*	196.3±19.4*	205.7±19.1*	190.7±18.9*	<0.01
AST (U/L)	40.1±5.2	195.3±16.1*	202.0±17.3*	188.8±17.0*	191.4±15.2*	<0.01
GGT (U/L)	0.6±0.2	3.9±0.7*	4.1±0.6*	3.8±0.4*	3.9±0.6*	<0.01
ALP (U/L)	232.7±18.9	408.7±21.6*	423.3±19.8*	412.7±23.2*	431.6±19.3*	<0.01
TG (mmol/L)	0.9±0.1	2.7±0.2*	2.5±0.3*	2.5±0.3*	2.6±0.2*	<0.01
TC (mmol/L)	6.3±0.3	13.2±1.2*	11.8±0.9*	12.2±1.0*	12.4±1.1*	<0.01
HDL-C (mmol/L)	5.2±0.3	5.1±0.2	5.5±0.4	5.2±0.2	5.3±0.4	0.51
LDL-C (mmol/L)	3.6±0.2	5.5±0.7*	5.1±0.8*	4.8±0.6*	4.9±0.6*	<0.01
Glu (mmol/L)	7.7±0.3	10.2±0.4*	9.8±0.5*	10.5±0.5*	10.3±0.6*	<0.01

Table 1: Baseline DHBV in sera, and lipids and liver enzymes measured in the serum of ducks in all groups.
**indicates significant difference compared with sham group. E Group: entecavir group; A group: atorvastatin group; E&A group: entecavir and atorvastatin group. ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, glutamyl transpeptidase; ALP, alkaline phosphatase. TG, triglyceride; TC, cholesterol; HDL-C, high-density lipoprotein cholesterin; LDL-C, low-density lipoprotein cholesterin; GLU, glucose.*

Liver enzymes (ALT, AST, GGT, and ALP) and most lipid metabolism parameters (TG, TC, LDL-C, and Glu) were significantly different in five groups (all P<0.01), post-hoc analyses indicated that these parameters in all ducks of NAFLD model were significantly higher than those in the sham group.

Figure 1 displays the changes in liver enzyme levels of the five groups from Day 0 to Day 21. Repeated measures ANOVA showed that ALT, AST, GGT, and ALP were significantly different among five groups (all P<0.01), post-hoc analyses indicated that all liver enzyme levels in the E&A group were significantly lower than those in the E group, A group, and control group; these parameters in the A group were significantly lower than those in the E group and control group.

Figure 2 shows the changes in lipid metabolism parameters of five groups from Day 0 to Day 21. Repeated measures ANOVA showed that TG, TC, LDL-C, and Glu were significantly different among five groups (all P<0.01), post-hoc analyses indicated that all lipid metabolism parameters in the E&A group and A group were significantly lower than those in the E group and control group; the differences in these parameters between E&A group, and A group were not significant.

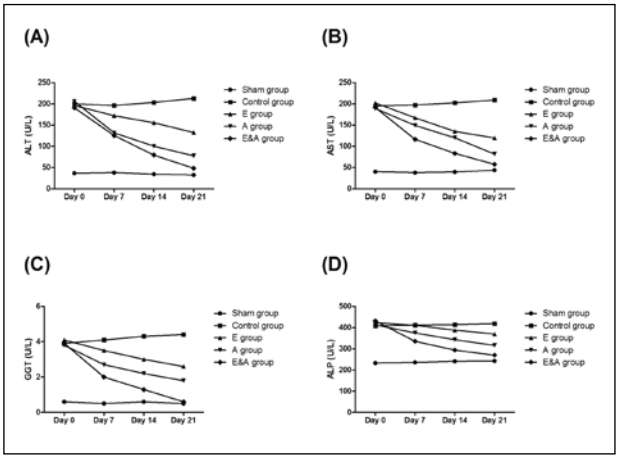

Figure 1: Liver enzyme levels including ALT (a), AST (b), GGT (c), and ALP (d) on day 0, day 7, day 14 and day 21.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, glutamyl transpeptidase; ALP, alkaline phosphatase.

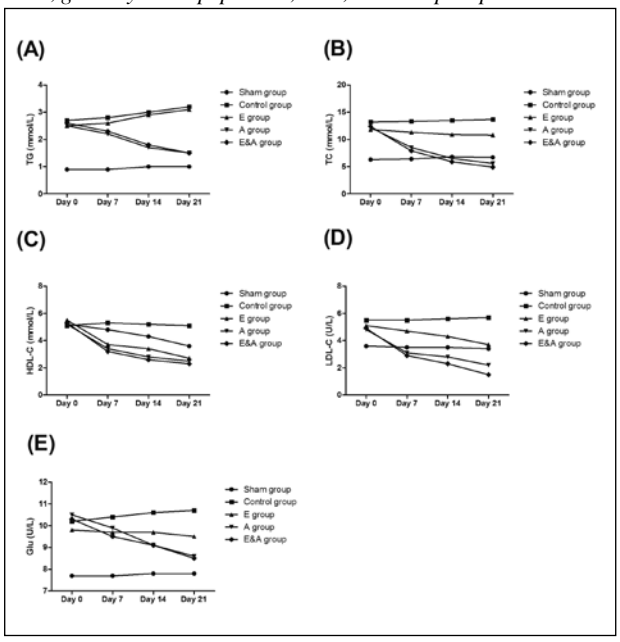

Figure 2: Lipid metabolism parameter levels including TG (a), TC (b), HDL-C (c), LDL-C (d), and Glu (e) on day 0, day 7, day 14 and day 21.
TG, triglyceride; TC, cholesterol; HDL-C, high-density lipoprotein cholesterin; LDL-C, low-density lipoprotein cholesterin; GLU, glucose.

Table 2 shows that DHBV levels in serum and liver of ducks were significantly different among five groups (both P<0.01), post-hoc analyses indicated that DHBV levels in serum and liver in the E&A group and E group were significantly lower than those in the A group, control group and sham group; and DHBV levels in the E&A group was significantly lower than those in the E group.

	Sham group	Control group	E group	A group	E&A group	*P*
DHBV in sera	8.9±0.7	9.0±0.6	5.3±0.4*	8.8±0.8	3.6±0.3*#	<0.01
DHBV in liver	8.6±0.6	8.8±0.7	6.1±0.5*	9.1±0.9	4.8±0.4*#	<0.01

Table 2: DHBV levels in sera and livers of ducks in all groups.
**indicates significant difference compared with sham group, control group and A group. #indicates significant difference compared with E group. E Group: entecavir group; A group: atorvastatin group; E&A group: entecavir and atorvastatin group.*

Discussion and conclusion

CHB and NAFLD are two major causes of chronic liver disorders that undermine liver function and potentiate end-stage liver diseases such as hepatocellular carcinoma[2]. This study successfully established a duck model of CHB and NAFLD and indicated that liver function and lipid metabolism parameters, as well as serum and intrahepatic DHBV DNA levels were significantly lower in ducks treated with entecavir and atorvastatin.

Antiviral strategy is widely employed to prevent the progression of CHB-related liver diseases and the occurrence of hepatocellular carcinoma. Entecavir, a cyclopentyl 2'-deoxyguanosine nucleoside, is one of the most promising novel agents that display potent and selective inhibition of HBV[11, 12]. It is reported that entecavir contributes to the inhibition of hepadnaviral polymerase and suppression of HBV replication[13]. In line with previously reported studies in the duck model of HBV, our present study demonstrated that DHBV levels in serum and liver in the E&A group and E group were significantly lower than those in the A group, control group, and sham group, indicating that entecavir effectively inhibited the HBV replication in ducks.

Atorvastatin is a synthetic competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which catalyzes the conversion of HMG-CoA to mevalonate. This reaction serves as an early and rate-limiting step in cholesterol biosynthesis, leading to depletion of the intracellular supply of cholesterol[14]. Several studies reveal that treatment with atorvastatin is effective and safe for animals and patients with NAFLD[15, 16]. Our study also showed that atorvastatin alone or a combination of entecavir and atorvastatin significantly reduced the lipid metabolism parameter levels and liver enzyme levels. An increasing number of evidence showed that a compromised therapeutic effect of antiviral treatment under hepatic steatosis. A nested case-control research involving 267 CHB patients implied that those with hepatic steatosis were prone to nonresponse to entecavir[17]. A meta-analysis also indicated that hepatic steatosis could diminish the effect of antiviral therapy in CHB patients[18]. A Taiwan study revealed that high HBV load was negatively associated with hypertriglyceridemia (Odd ratio=0.74, 95% Confidence Interval: 0.61-0.89) in HBV-infected participants[19]. Although the association of hepatic steatosis with antiviral therapy is still debatable, the onset or progression of NAFLD during antiviral treatment remains recommended to be monitored in case of underlying negative impact[2]. Our study demonstrated that, compared with entecavir, the combination of entecavir and atorvastatin should be a more promising therapy to inhibit the replication of DHBV through improving hepatic steatosis.

In conclusion, the combination of entecavir and atorvastatin could significantly improve hepatic steatosis and increase antiviral responses in the duck model of HBV and NAFLD.

References

1) Te HS, Jensen DM. Epidemiology of hepatitis B and C viruses: a global overview. Clin Liver Dis. 2010; 14(1): 1-21, VII.
2) Zhang J, Lin S, Jiang D, Li M, Chen Y, Li J, et al. Chronic hepatitis B and non-alcoholic fatty liver disease: Conspirators or competitors? Liver Int. 2020; 40(3): 496-508.
3) Angulo P. Nonalcoholic fatty liver disease. N Engl J Med. 2002; 346(16): 1221-31.
4) Takahashi Y, Soejima Y, Fukusato T. Animal models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. World J Gastroenterol. 2012; 18(19): 2300-8.
5) Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. Hepatology. 2016; 64(1): 73-84.
6) Chalasani N, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, et al. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. Hepatology. 2018; 67(1): 328-57.
7) Charatcharoenwitthaya P, Pongpaibul A, Kaosombatwattana U, Bhanthumkomol P, Bandidniyamanon W, Pausawasdi N, et al. The prevalence of steatohepatitis in chronic hepatitis B patients and its impact on disease severity and treatment response. Liver Int. 2017; 37(4): 542-51.

8) Choi HSJ, Brouwer WP, Zanjir WMR, de Man RA, Feld JJ, Hansen BE, et al. Nonalcoholic Steatohepatitis Is Associated With Liver-Related Outcomes and All-Cause Mortality in Chronic Hepatitis B. Hepatology. 2020; 71(2): 539-48.
9) European Association for the Study of the Liver. Electronic address eee, European Association for the Study of the L. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. J Hepatol. 2017; 67(2): 370-98.
10) Martin-Castillo A, Castells MT, Adanez G, Polo MT, Perez BG, Ayala I. Effect of atorvastatin and diet on non-alcoholic fatty liver disease activity score in hyperlipidemic chickens. Biomed Pharmacother. 2010; 64(4): 275-81.
11) Marion PL, Salazar FH, Winters MA, Colonno RJ. Potent efficacy of entecavir (BMS-200475) in a duck model of hepatitis B virus replication. Antimicrob Agents Chemother. 2002; 46(1): 82-8.
12) Colonno RJ, Genovesi EV, Medina I, Lamb L, Durham SK, Huang ML, et al. Long-term entecavir treatment results in sustained antiviral efficacy and prolonged life span in the woodchuck model of chronic hepatitis infection. J Infect Dis. 2001; 184(10): 1236-45.
13) Seifer M, Hamatake RK, Colonno RJ, Standring DN. In vitro inhibition of hepadnavirus polymerases by the triphosphates of BMS-200475 and lobucavir. Antimicrob Agents Chemother. 1998; 42(12): 3200-8.
14) Taylor F, Ward K, Moore TH, Burke M, Davey Smith G, Casas JP, et al. Statins for the primary prevention of cardiovascular disease. Cochrane Database Syst Rev. 2011(1): CD004816.
15) Ji G, Zhao X, Leng L, Liu P, Jiang Z. Comparison of dietary control and atorvastatin on high fat diet induced hepatic steatosis and hyperlipidemia in rats. Lipids Health Dis. 2011; 10:23.
16) Kimura Y, Hyogo H, Yamagishi S, Takeuchi M, Ishitobi T, Nabeshima Y, et al. Atorvastatin decreases serum levels of advanced glycation endproducts (AGEs) in nonalcoholic steatohepatitis (NASH) patients with dyslipidemia: clinical usefulness of AGEs as a biomarker for the attenuation of NASH. J Gastroenterol. 2010; 45(7): 750-7.
17) Jin X, Chen YP, Yang YD, Li YM, Zheng L, Xu CQ. Association between hepatic steatosis and entecavir treatment failure in Chinese patients with chronic hepatitis B. PLoS One. 2012; 7(3): e34198.
18) Zhu Y, Yang Q, Lv F, Yu Y. The Effect of Hepatosteatosis on Response to Antiviral Treatment in Patients with Chronic Hepatitis B: A Meta-Analysis. Gastroenterol Res Pract. 2017; 2017: 1096406.
19) Chiang CH, Yang HI, Jen CL, Lu SN, Wang LY, You SL, et al. Association between obesity, hypertriglyceridemia and low hepatitis B viral load. Int J Obes (Lond). 2013; 37(3): 410-5.

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