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The anti-DHAV activities of *Astragalus* polysaccharide and its sulfate compared with those of BSRPS and its sulfate

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ABSTRACT

This paper studied the anti-duck hepatitis A virus (DHAV) activities of Astragalus polysaccharide (APS) and its sulfate (sAPS) compared with those of Bush Sophora Root polysaccharide (BSRPS) and its sulfate (sBSRPS). The antiviral activities of APS and sAPS were measured by MTT and real-time PCR methods, in vitro. In vivo experiment, the mortality rate and the evaluation indexes of hepatic injury, peroxidative injury and immune level were measured. Just like the condition of BSRPS and sBSRPS, the anti-DHAV activities of sAPS were stronger than those of APS, both in vitro and in vivo. It indicated sulfated modification could enhance the antiviral ability of polysaccharide. But unlike the antiviral effects of BSPRS and sBSRPS in vivo, APS and sAPS did not reduce the mortality rates as their abilities of scavenging free radicals and alleviating the hepatic injuries were weaker than those of BSRPS and sBSRPS. And they even did not enhance the immune levels.

1. Introduction

Duck virus hepatitis (DVH) caused by duck hepatitis A virus (DHAV) still damages the interest of duck industry. Our previous study certifies that this disease is related to the levels of immune and peroxidative injury of bodies (Chen, Xiong, Zeng, Wang, Zhang, et al., 2014). Bush Sophora Root polysaccharide (BSRPS) and its sulfate, sulfated Bush Sophora Root polysaccharide (sBSRPS), exhibit excellent antiviral activities against DHAV (Chen, Xiong, Zeng, Wang, Liu, et al., 2014;Chen, Xiong, Zeng, Wang, Zhang, et al., 2014). BSRPS and sBSRPS can inhibit the virus proliferations (inhibition rates are 53.38% and 58.25%, respectively) and gene replications (inhibition rates are 55.8% and 32.1%, respectively), in vitro experiment. They also can alleviate the hepatic pathological injury severities and reduce the mortality rates, in vivo experiment. The anti-DHAV effects of BSRPS and its sulfate are related to their immuno-enhancing and antioxidant activities (Chen, Xiong, Zeng, Wang, Zhang, et al., 2014).

Astragalus polysaccharide (APS) also owns antioxidant (Li, Chen, Wang, Tian, & Zhang, 2010) and immuno-enhancing (Wang et al., 2009; Yin et al., 2010) effects. APS is a primary active ingredient of the extraction from *Astragalus membranaceus* which is a frequently-used and widely-known Chinese herbal medicine used for tonifying Qi, strengthening exterior, etc. The average molecular weight of APS was 1334 kDa composing of rhamnose, arabinose, glucose, galactose and galacturonic acid in a molar ratio of 0.03:1.00:0.27:0.36:0.30 (Yin et al., 2012). It is extensively used in the clinic resulting from its multiple functions of immuno-enhancing (Wang et al., 2009; Yin et al., 2010), resisting immunosuppression (Guo et al., 2012), antiviral (Li, Lloyd, & Wang, 2007), antioxidant (Li et al., 2010) and so on activities. Sulfated modification as a molecular modification method can enhance the immuno-enhancing (Wang et al., 2010) and antiviral (Chen, Xiong, Zeng, Wang, Liu, et al., 2014;Chen, Xiong, Zeng, Wang, Zhang, et al., 2014) activities of polysaccharides. The study of Huang et al. indicates that sulfated modification can enhance the adjuvant activity

Abbreviations: DVH, duck virus hepatitis; DHAV, duck hepatitis A virus; BSRPS, Bush Sophora Root polysaccharide; sBSRPS, sulfated Bush Sophora Root polysaccharide; APS, Astragalus polysaccharide; sAPS, sulfated Astragalus polysaccharide; DMEM, Dulbecco's modified eagle medium; DEHs, duck embryonic hepatocytes; MM, maintenance medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CMF-PBS, calcium and magnesium-free phosphate-buffered saline; CC, cell control; VC, virus control; BC, blank control; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; SOD, superoxide dismutase; MDA, malondialdehyd; CAT, catalase; GSH-Px, glutathione peroxidase; NOS, nitric oxide synthase.

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of APS for newcastle disease vaccine (Huang, Hu, et al., 2008). Therefore, APS is expected to be a kind of antiviral drug against DHAV and sulfated modification is a pregnant modification method predictably.

In the present research, APS and sulfated *Astragalus* polysaccharide (sAPS) were prepared by the water decoction, the ethanol precipitation method and the chlorosulfonic acid-pyridine method, respectively. Anti-DHAV effects experiments in vitro and in vivo were conducted, and all the indexes of APS and its sulfate were compared with those of BSRPS and sBSRPS, whose aim is to ascertain the antiviral effects of APS and its sulfate and their possibilities of being anti-DVH drugs.

2. Materials and methods

2.1. Reagents

Dulbecco's modified eagle medium (DMEM) (Gibco) supplemented with penicillin 100 IU/mL, streptomycin 100 IU/mL, glutamine 0.75 mg/mL and 10% fetal bovine serum, was called growth medium and used for culturing the duck embryonic hepatocytes (DEHs); 1% fetal bovine serum, maintenance medium (MM) for diluting drugs and maintaining cells. D-Hank's solution was used for washing the embryo tissue and cells. DMEM, MM and D-Hank's, pH was adjusted to 7.4 using 5.6% $NaHCO_3$. The trypsin (Amresco) was dissolved into 0.20% with D-Hank's. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Amresco) was dissolved with calcium and magnesium-free phosphate-buffered saline (CMF-PBS, pH 7.4) into 5 mg/mL. Heparin sodium was dissolved into 2 mg/mL with physiological saline and used as anticoagulant.

Pyridine (Lot no. 20130220) and chlorsulfonic acid (Lot no. 130622) were the products of Sinopharm Group Chemical Company and Shanghai Ling Feng Chemical Company, respectively. RNAiso Plus Reagent (Lot no. AK7202), PrimeScript™ RT Master Mix Kit (Lot no. AK2702) and SYBR® Premix Ex Taq™ (Tli RNaseH Plus) Kit (Lot no. AK3802) were bought from Takara.

2.2. Drugs and virus

APS was prepared by the water decoction and the ethanol precipitation method (Huang, Hu, et al., 2008). *Astragalus membranaceus* was decocted three times within 10-fold water, 1.5 h per time. The decoctions were merged together and condensed into 1 g materia medica/mL. The impurities of condensation were removed by centrifugation, subsequently. Ninety-five percent ethanol was added into the supernatant to make ethanol concentration reach to 75% (v/v). Then, the APS was gained from the sediment and was dried at 60 °C after 24 h. It was purified by the Sevage method and column chromatography of Sephadex G-200 (Qin et al., 2013) and the polysaccharide content was 93.96% determined by the phenol–sulfuric acid method (Hsieh, Hsu, & Yang, 2005).

Chlorosulfonic acid–pyridine method was applied to prepare sAPS (Huang, Hu, et al., 2008). Chlorsulfonic acid and pyridine were mixed together in an ice bath according to the ratio of 1:6. Four hundred milligram APS was added into the above-mentioned mixture (21 mL) under a reaction condition of 95 °C and 1 h with stirring. And then the solution was neutralized with NaOH, dialyzed and lyophilized to sAPS, successively. The content of sAPS was 99.06% calculated with the sum of its polysaccharide content determined by the phenol–sulfuric acid method and sulfur content determined by the barium chloride–gelatin method (Dodgson & Price, 1962).

DHAV (LQ_2 strain) was supplied by the Shandong Institute of Poultry in China.

2.3. Anti-DHAV effect in vitro

2.3.1. Duck embryonic hepatocytes (DEHs)

DEHs were prepared as the method described previously (Chen, Xiong, Zeng, Wang, Liu, et al., 2014). Briefly, hepar tissues from a duck embryonic were minced and then digested with 0.20% trypsin. They were then washed thrice with D-Hank's and cultured into the DMEM in a humid atmosphere of 5% CO_2 at 37 °C. After 48 h, the hepatocytes grew into a monolayer and were taken for standby.

2.3.2. Antiviral activities

APS was twofold diluted into four concentrations, from 625 μg/mL to 78.125 μg/mL, with MM according to the predict experiment (cytotoxicity test) and taken for standby, sAPS from 7.813 μg/mL to 0.977 μg/mL.

The 96-well plate containing a DEHs monolayer was divided into cell control (CC) group, virus control (VC) group, APS group and sAPS group. One hundred microliter DHAV was added into each well of the VC, the APS and the sAPS groups, and 100 μL MM was added into each well of the CC group, five wells per group. Then, the plate was incubated in a humid atmosphere of 5% CO_2 at 37 °C for 2 h. The DHAV and MM were subsequently removed and the 96-well plate was washed trice by D-Hank's. After that, 100 μL APS and sAPS at series concentration were added into the APS and the sAPS treated wells, respectively. And 100 μL MM was added into each well of the CC and the VC groups. The MTT colorimetric method (Zhang et al., 2012) was applied to measure the cytoactive after the plate being incubated in a humid atmosphere of 5% CO_2 at 37 °C for 96 h. The virus inhibitory rate was calculated based on the formula: Virus inhibitory rate $(\%) = (\bar{A}_{\text{drug+virus}} - \bar{A}_{\text{virus control}})/(\bar{A}_{\text{cell control}} - \bar{A}_{\text{virus control}}) \times 100\%$ (Takeuchi, Baba, & Shigeta, 1991).

2.3.3. Assay of virus replication on DEHs

APS and sAPS were diluted into the most effective antiviral concentration with MM according to the results of Section 2.3.2, respectively.

Four hundred microliters DHAV was added into each well of the VC, the APS and the sAPS groups of the 24-well plate containing a DEHs monolayer; 400 μL MM was added into each well of the CC group, three wells per group. The plate was incubated in a humid atmosphere of 5% CO_2 at 37 °C for 2 h. Then the solutions were removed and the 24-well plate was washed trice by D-Hank's. 400 μL APS, sAPS and MM were added into the corresponding wells, respectively. Total RNA was extracted after the plate being incubated for 24 h by RNAiso Plus Reagent. And reverse transcription was immediately performed using PrimeScript™ RT Master Mix Kit. Finally, real-time PCR was used to semi-quantitative analysis the virus replication by SYBR® Premix Ex Taq™ (Tli RNaseH Plus) Kit. The primers of DHAV (forward, 5′-GCCACCCTTCCTGAGTTTGT-3′; reverse, 5′-TACCATTCCACTTCTCCTGCTT-3′) and β-actin (forward, 5′-CTTTCTTGGGTATGGAGTCCTG-3′; reverse, 5′-TGATTTTCATCGTGCTGGGT-3′) were designed as the method described previously (Chen, Xiong, Zeng, Wang, Liu, et al., 2014)

2.4. Animals grouping and treatment

A total of 240 four-day-old cherry valley ducklings (Purchased from Tangquan Poultry Farm, Jiangsu province, China) were randomly divided into APS, sAPS, VC and blank control (BC, separately reared) groups. The ducklings in the APS, the sAPS and the VC groups were intramuscularly injected DHAV 0.2 mL per feather, the BC group treated with physiological saline in the meantime. The ducklings in the APS and the sAPS groups were treated with aqueous APS and sAPS solution, respectively, both at the dosage of 3 mg per feather according to the net content, once a day for 5 days. In order