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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

Table 1
The A_{570} values and virus inhibitory rates.

Group	Concentration (μg/mL)	A_{570}	Virus inhibitory rate (%)	Group	Concentration (μg/mL)	A_{570}	Virus inhibitory rate (%)
APS	625	0.325 ± 0.007^{c}	14.29	sAPS	7.813	0.393 ± 0.006^{cd}	48.98
	312.5	0.403 ± 0.005^{b}	54.08		3.906	0.413 ± 0.015^{c}	59.18
	156.25	0.389 ± 0.014^{b}	46.94		1.953	0.456 ± 0.008^{b}	81.12
	78.125	0.327 ± 0.014^{c}	15.31		0.977	0.360 ± 0.002^{d}	32.14
VC		0.297 ± 0.021^{c}		VC		0.297 ± 0.021^{e}	
CC		0.493 ± 0.006^{a}		CC		0.493 ± 0.006^{a}	

[a–e] Data within a column without the same superscripts (a–e) differ significantly ($p < 0.05$).

to monitor the evaluation indexes of hepatic injury and immune level at the 4th, the 8th and the 54th hour, peroxidative injury at the 8th and the 54th hour, the blood samples were taken from five ducklings per group. The numbers of these ducklings (15 feathers per group) were not calculated in the mortality rate.

Once duck death was found, the pathology changes would be examined carefully and the number of the ducklings without pathology changes would be eliminated. The numbers of deaths were recorded at the 12th, the 24th, the 36th, the 48th, the 72nd, the 96th, the 120th and the 144th hour (Chen, Xiong, Zeng, Wang, Zhang, et al., 2014) after challenged virus, and the mortality rate of each group was calculated until no death was found. Survival ducklings after determining rehabilitation were sent to Nanjing Tangquan Poultry Farm to be bred in accordance with National regulation. Dead ducklings were executed bio-safety disposal according to local standard protocols.

2.5. Evaluation indexes of hepatic injury, peroxidative injury and immune level

The serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) at the 4th, the 8th and the 54th hour were tested by enzymatic colorimetry using automatic biochemistry analyzer (7180 Automatic Analyzer, HITACHI, Japan) in Nanjing Shihuang Institute of Animal Science and Technology. The superoxide dismutase (SOD), malondialdehyd (MDA), catalase (CAT), glutathione peroxidase (GSH-Px) and nitric oxide synthase (NOS) in ducklings' serum at the 8th and the 54th hour were determined by ELISA kits (BioCalvin, China); anti-DHAV Ab, IL-2 and IFN-γ at the 4th, the 8th and the 54th hour by ELISA kits (BioCalvin, China).

2.6. Statistical analysis

Data were expressed as means ± S.E. Statistical analyses were performed by Duncan's multiple range test or χ^2 test using SPSS Software Package v.20.0. $2^{-\Delta\Delta CT}$ method (Kenneth & Thomas, 2001) was used to analysis the relative gene expression data. Differences between means were considered significant at $p < 0.05$.

3. Results

3.1. Antiviral activity on DEHs

As the results listed in Table 1, only two A_{570} values in the APS group (concentrations at 312.5 μg/mL and 156.25 μg/mL) were significantly higher than that in the VC group ($p < 0.05$), while all the A_{570} values in the sAPS group were significantly higher ($p < 0.05$). The maximum virus inhibitory rate of APS (312.5 μg/mL) was 54.08% and lower than that of sAPS (1.953 μg/mL, 81.12%).

3.2. Virus replication on DEHs

Fig. 1 shows the relative DHAV gene expression in each group. No virus gene expression was detected in the CC group. The DHAV gene expression in the VC group was set to 1. The relative DHAV gene expressions in the APS and the sAPS groups were 0.404 and 0.210, respectively. The expressions in these two groups were significantly lower than that in the VC group ($p < 0.05$), and the expression in the sAPS group was significantly lower than that in the APS group ($p < 0.05$).

3.3. Therapeutic effects

Fig. 2 illustrates the dynamic death in each group. The ducklings in the BC group were healthy and had no death. The ducklings in the APS, the sAPS and the VC groups stopped the death at the 120th hour. The death peak of ducklings in the VC group was around the 24th hour, and around the 72nd hour a small dying peak appeared. Meanwhile, two peaks of dying appeared in both the APS and the sAPS group: the first peak emerged at the 24th hour; the second small peak emerged at the 72nd and the 96th hour. Before the 60th hour, the numbers of deaths in the APS (32 feathers) and the sAPS

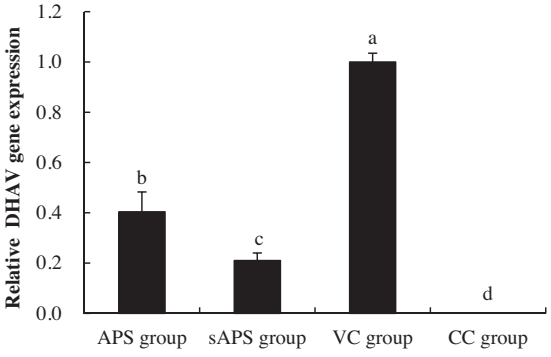

Fig. 1. The effects of APS and sAPS on DHAV replications measured by real-time PCR assay.

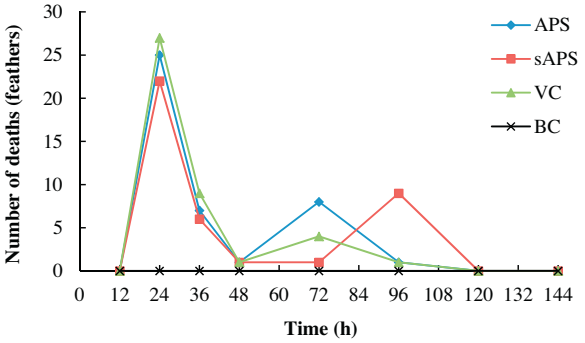

Fig. 2. The dynamic death of each group.

Table 2
The evaluation indexes of hepatic injury.

	Time (h)	APS	sAPS	VC	BC
ALT (IU/L)	4	26.3 ± 2.1^{a}	33.2 ± 6.1^{a}	32.0 ± 5.7^{a}	23.4 ± 2.1^{a}
	8	35.0 ± 1.4^{a}	36.4 ± 2.8^{a}	36.2 ± 3.8^{a}	26.6 ± 1.2^{b}
	54	54.7 ± 1.9^{ab}	46.3 ± 1.5^{ab}	66.0 ± 12.4^{a}	28.0 ± 1.0^{b}
AST (IU/L)	4	15.0 ± 1.3^{b}	14.0 ± 1.4^{b}	34.4 ± 9.4^{a}	15.6 ± 0.9^{b}
	8	19.2 ± 1.6^{a}	17.8 ± 2.8^{a}	18.3 ± 1.7^{a}	17.2 ± 2.1^{a}
	54	115.0 ± 14.0^{a}	19.2 ± 1.7^{b}	121.3 ± 55.3^{a}	12.8 ± 0.4^{b}
ALP (IU/L)	4	585.2 ± 37.5^{b}	622.5 ± 23.8^{b}	849.3 ± 81.7^{a}	669.6 ± 30.3^{b}
	8	760.6 ± 38.1^{a}	708.6 ± 22.9^{a}	677.5 ± 30.4^{a}	535.8 ± 66.4^{b}
	54	1114.6 ± 85.6^{a}	1258.0 ± 87.6^{a}	1031.5 ± 108.7^{a}	679.8 ± 50.8^{b}
LDH (IU/L)	4	382.4 ± 27.2^{a}	435.0 ± 53.8^{a}	455.3 ± 32.4^{a}	353.8 ± 10.5^{a}
	8	401.8 ± 19.5^{a}	400.5 ± 80.6^{a}	429.3 ± 41.8^{a}	354.6 ± 37.2^{a}
	54	4597.5 ± 208.5^{b}	1302.0 ± 90.0^{c}	5695.0 ± 342.0^{a}	244.2 ± 9.8^{d}

[a–d] Data within a row without the same superscripts (a–d) differ significant ($p < 0.05$).

(28 feathers) groups, especially in the sAPS group, were both lower than that in the VC groups (36 feathers). However, the death numbers in the APS and the sAPS groups in the second peak were higher than that in the VC group, and the ultimate mortality rates in these three groups were very nearly the same (93.33%, 86.67% and 93.33%, respectively).

3.4. Results of evaluation indexes of hepatic injury

The evaluation indexes of hepatic injury at the 4th, the 8th and the 54th hour are listed in Table 2.

The ALT levels of ducklings in the APS, the sAPS and the VC groups were at the same levels with that in the BC group at the 4th hour and significantly increased at the 8th hour ($p < 0.05$). At the 54th hour, the ALT level in the VC group was significantly higher than that in the BC group ($p < 0.05$); ALT levels in the APS and the sAPS groups were both lower than that in the VC group and higher than that in the BC group but without any significance.

At the 4th hour, the AST level in the VC group was significantly higher than those in the APS, the sAPS and the BC groups ($p < 0.05$). At the 8th hour, the AST levels were the same. At the 54th hour, the AST levels in the APS and the VC groups both significantly augmented ($p < 0.05$) compared with those in the sAPS and the BC groups.

At the 4th hour, the ALP value in the VC group was significantly higher than those in another three groups ($p < 0.05$). At the 8th and the 54th hour, the ALP values in the BC group were significantly lower than those in another three groups ($p < 0.05$).

The LDH levels in the four groups were consistent at the 4th and the 8th hour. At the 54th hour, the highest level appeared in the VC group, and then following the APS, the sAPS and the BC groups in order. The difference between each other was significant ($p < 0.05$).

3.5. Results of evaluation indexes of peroxidative injury

Fig. 3 displays the serum levels of SOD, MDA, CAT, GSH-Px and NOS in each group at the 8th and the 54th hour.

There was no distinction at the MDA or the NOS level between each other at the 8th hour. And the SOD activity of ducklings in the sAPS group significantly rose ($p < 0.05$), GSH-Px activity declined, as comparison with those in the other three groups. The CAT activity in the APS group was the same as those in the VC and the BC groups while significantly higher ($p < 0.05$) than that in the sAPS group.

Compared with those in the BC groups at the 54th hour, the SOD, CAT and GSH-Px levels in the VC groups were significantly decreased ($p < 0.05$), also did SOD level in the sAPS group and CAT level in the APS group, MDA and NOS levels increased. The SOD, MDA, GSH-Px and NOS values in the APS group were at the BC value, and the MDA, CAT GSH-Px and NOS values in the sAPS group were at the BC value at this time point. Though the GSH-Px activities in the APS and the sAPS groups were at the same level with that in the BC groups, they were indistinctively higher than that in the VC group.

3.6. Results of evaluation indexes of immune level

The anti-DHAV Ab, IFN-γ and IL-2 levels in each group are listed in Table 3.

At the 4th hour, the value of Ab significantly went up in the VC and the sAPS groups ($p < 0.05$), as comparison with that in the BC group. The value of Ab in the APS group was higher than that in the BC group, lower than that in the sAPS group, but without a significant difference, significantly lower than that in the VC group ($p < 0.05$). The Ab in the VC group still maintained a high level at the 8th hour, while the Ab in the sAPS group reduced to the BC level. And even the value of Ab in the APS group significantly lower than that in the BC group ($p < 0.05$). At the 54th hour, the values of Ab in all groups were the same.

There was not much difference between the IFN-γ levels of each other when at the 4th and the 54th hour. At the 8th hour, the IFN-γ levels in the APS, the sAPS and the VC groups rose, especially in the APS and the VC groups ($p < 0.05$), as comparison with that in the BC group.

The IL-2 of each group was at the same level in all time.

4. Discussion

APS has a strong antiviral effect. Huang et al. studied the anti-infectious bursal disease virus activity of APS in vitro (Huang, Wang, et al., 2008), and the results showed the A_{570} values of APS (from 4.883 to 1.221 μg/mL) were 0.375, 0.345 and 0.225 which significantly higher ($p < 0.05$) than that of VC (0.141). The A_{570} values measured in MTT method are positively correlated with cytoactivities (Verma et al., 2010). It made clear the antiviral activity of APS in vitro. APS also presented a good anti-DHAV effect in present research. The A_{570} values of APS (312.5 and 156.25 μg/mL) were 0.403 and 0.389 while that of VC was 0.297. Certainly, the differences were significant ($p < 0.05$). Sulfated modification enhanced the antiviral activity and led to higher A_{570} values of all concentrations (Table 1). In the study about BSRPS and sBSRPS, the A_{570} values of all concentrations of both two drugs were significantly higher than that of VC ($p < 0.05$) and the difference of sBSRPS was more significant (Chen, Xiong, Zeng, Wang, Liu, et al., 2014). It meant after the modification, sAPS acted out better anti-DHAV activity with wider range of concentration and sBSRPS exhibited better anti-DHAV activity. The highest virus inhibitory rate of APS (54.08%) was similar to that of BSRPS (Chen, Xiong, Zeng, Wang, Liu, et al., 2014, 53.38%), while that of sAPS (81.12%) was higher than that of sBSRPS (Chen, Xiong, Zeng, Wang, Liu, et al., 2014, 58.25%). It showed that sulfated modification can enhance the antiviral

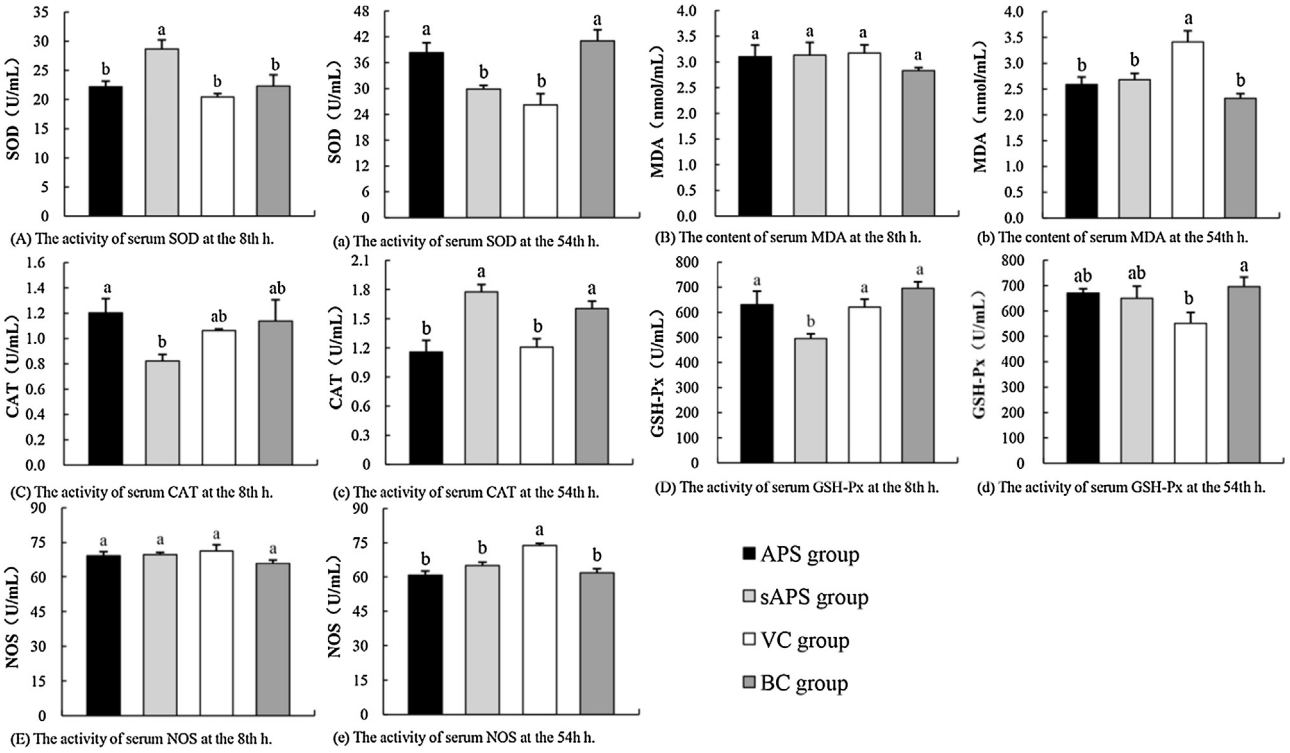

Fig. 3. The evaluation indexes of peroxidative injury at the 8th and the 54th hour.

activity of polysaccharide and the potentiation of anti-DHAV ability of sulfated modification was more obvious to APS, as comparison with BSRPS, in vitro. Not only the virus inhibitory rates but also the gene replications expound the anti-DHAV activity (Fig. 1). After cultured under APS environment for 24 h, the DHAV gene expression reduced to 40.4% of VC. Obviously, the significant lower ($p < 0.05$) expression certified that sAPS did better in suppressing virus gene replications. The relative DHAV gene expressions of BSRPS and its sulfate were 0.442 and 0.679, respectively (Chen, Xiong, Zeng, Wang, Liu, et al., 2014). It signified after the modification, the ability of inhibiting DHAV gene expression of sAPS heightened, sBSRPS receded. The above indicated that APS exhibited the anti-DHAV activity, sulfated modification could enhance the anti-DHAV activity of APS more effectively compared with BSRPS, in vitro.

After the treatment of APS and sAPS, however, the mortality rates did not reduce much compared with that of the ducklings which got neither drug (Fig. 2.). In our previous study, BSRPS and sBSRPS indeed reduced the mortality rates (Chen, Xiong, Zeng, Wang, Zhang, et al., 2014). The significant lower deaths ($p < 0.05$) in the period of death peak in the BSRPS and the sBSRPS groups led to the significant lower mortality rates, compared with the VC group (Chen, Xiong, Zeng, Wang, Zhang, et al., 2014). In the present research, in the first death peak, the death numbers of ducklings in the APS and the sAPS groups really declined, too (Fig. 2). However, in the second death peak, the death numbers were more than that in the VC group, although the sulfate postponed the time of the second death peak (Fig. 2). Unlike the significant lower ultimate mortality rates in the BSRPS and the sBSRPS groups (Chen, Xiong, Zeng, Wang, Zhang, et al., 2014), those in the APS and the sAPS groups were not lower or significantly lower than that in the VC group. At the same time the numbers of deaths in the APS and the sAPS groups in every time point in the first death peak was always lower than those in the VC group, which was the same as BSRPS and sBSRPS (Chen, Xiong, Zeng, Wang, Zhang, et al., 2014). Therefore, it could deduce that APS and its sulfate subserved the treatment in the acute stage of DVH and sAPS could buy more time for the treatment of DVH.

When infected with acute or chronic hepatitis, the levels of ALT, AST, ALP and LDH in the body increase to some extent (Bourne et al., 2005; Elinav et al., 2005; Korachi, Ceran, Adaleti, Nigdelioglu, & Sökmen, 2013; Prati et al., 2002; Shin et al., 2008). These four indexes reflected the degree of hepars damage, directly or indirectly. Treated with BSRPS and sBSRPS, the hepatic injuries are alleviated at all time after challenging DHAV (Chen, Xiong, Zeng, Wang, Zhang, et al., 2014). It points out the mortality rates are closely linked to the hepatic injuries. After challenged by DHAV

Table 3
The evaluation indexes of immune level.

Index	Time (h)	APS	sAPS	VC	BC
Ab (U/mL)	4	20.9 ± 1.3^{bc}	24.2 ± 1.1^{ab}	27.3 ± 2.2^{a}	18.8 ± 0.6^{c}
	8	17.6 ± 1.4^{c}	21.5 ± 1.0^{b}	26.5 ± 0.4^{a}	23.0 ± 1.0^{b}
	54	22.0 ± 1.1^{a}	19.4 ± 2.8^{a}	20.3 ± 1.0^{a}	21.5 ± 0.5^{a}
IFN-γ (ng/L)	4	49.4 ± 2.0^{a}	48.4 ± 1.3^{a}	50.5 ± 1.7^{a}	46.5 ± 3.3^{a}
	8	49.8 ± 1.7^{a}	44.2 ± 2.9^{ab}	48.1 ± 1.8^{a}	40.7 ± 0.9^{b}
	54	46.9 ± 1.5^{a}	44.9 ± 2.6^{a}	46.4 ± 2.0^{a}	42.5 ± 1.8^{a}
IL-2 (ng/L)	4	190.8 ± 13.0^{a}	215.8 ± 18.6^{a}	223.7 ± 14.3^{a}	198.0 ± 10.8^{a}
	8	197.3 ± 8.2^{a}	232.2 ± 21.0^{a}	210.6 ± 27.7^{a}	185.5 ± 7.3^{a}
	54	216.3 ± 17.2^{a}	244.3 ± 29.1^{a}	234.3 ± 19.6^{a}	234.4 ± 30.8^{a}

[a–c] Data within a row without the same superscripts (a–c) differ significant ($p < 0.05$).

for 8 h and 54 h the ALT levels of ducklings in the VC group significantly went up, 4 h and 54 h the AST levels, 4 h, 8 h and 54 h the ALP levels, 54 h the LDH level (Table 2). The increments first appeared at the 4th hour and were very many at the 54th hour. It demonstrated the DVH was acute and serious. Only the levels of ALT and ALP at the 8th in the APS and the sAPS groups rose (Table 2). It indicated APS and sAPS could alleviate the acute injury of the hepar. And it might result in the fewer death numbers in the first peak period (Fig. 2). At the 54th hour, the level of LDH in the APS group significantly decreased ($p < 0.05$), as comparison with that in the VC group, but the amount was still much larger ($p < 0.05$) than that in the BC group. Another three indexes in the APS group were almost the same with those in the VC groups (Table 2). It suggested the weak ability of alleviating DVH of APS at this time and the nonnegligible relationship between this ability and the large death number in the second death peak (Fig. 2). Apparently, the hepatic injury was alleviated by sAPS at the 54th hour though serious compared with that of the BC group (Table 2), which bespake the reason of putting off the time of the second death peak (Fig. 2). Compared with APS and sAPS, the abilities of alleviating DVH of BSRPS and sBSRPS were stronger and more durable, which resulted in the significant lower mortality rates (Chen, Xiong, Zeng, Wang, Zhang, et al., 2014).

Virus hepatitis induces the increment of free radicals and the decrement of activities of antioxidase (Boya et al., 1999; Demirdag, Yilmaz, Ozdarendeli, Ozden, & Kalkan, 2003; Irshad, Chaudhuri, & Joshi, 2002; Sayal, Erçin, Erdil, Kartal, & Kayaaltı, 2013). At the 8th hour, the levels of free radicals in the BSRPS, sBSRPS, VC and BC groups are the same (Chen, Xiong, Zeng, Wang, Zhang, et al., 2014). It means DVH do not cause the peroxidative injury at the 8th hour. After infected with DHAV for 8 h, the peroxidative level in VC group did not change a lot; so did in the APS group (Fig. 3.). Interestingly, the activity of SOD significantly increased ($p < 0.05$) after the therapy of sAPS, GSH-Px significantly decreased ($p < 0.05$), and the activity of CAT in the sAPS group also reduced (Fig. 3). This kind of shift induced by sAPS might bring about the balance of free radicals, ultimately. At the 54th hour, numerous free radicals generated in the VC groups, and the antioxidase activities significantly declined (Fig. 3, $p < 0.05$). BSRPS and its sulfate can scavenge free radicals at the 54th hour resulted from duck virus hepatitis (Chen, Xiong, Zeng, Wang, Zhang, et al., 2014). Both APS and sAPS could also anesis the increment of free radicals as most of their evaluation indexes of peroxidative injury were at the BC level (Fig. 3). But, compared the puissant effects of scavenging free radicals of BSRPS and sBSRPS (Chen, Xiong, Zeng, Wang, Zhang, et al., 2014), the abilities of APS and sAPS were relatively feeble. Because the levels of CAT (significantly, $p < 0.05$) and GSH-Px in the APS group were declined, meanwhile the activities of SOD (significantly, $p < 0.05$) and GSH-Px in the sAPS group were declined. This phenomenon probably brought out the high mortality rates in the APS and sAPS groups.

BSRPS and sBSRPS can enhance the immune levels of ducklings when infected DHAV. It indicates immuno-enhancing effect is important to treat DVH. The immuno-enhancing ability of APS has been certified in many studies (Du et al., 2012; Wang et al., 2009; Yuan et al., 2008; Zhang et al., 2010). Zhang et al. drenched the APS at doses of 0.0, 6.25, 12.5, 25, 50 mg/kg to the BALB/c mice, one day before the immunization, led to the high titer of Ab (Zhang et al., 2010). Nevertheless, compared with that in the VC group, the values of Ab in the APS and the sAPS groups did not increased (Table 3). In the same way, the high levels of IFN-γ and IL-2 induced by APS is reported (Du et al., 2012). However, these high levels were not presented in the APS and sAPS groups, as comparison with those in the VC group (Table 3). It showed that APS and its sulfate could not enhance the immune response in ducklings infected with DHAV.

Just like BSRPS and sBSRPS, APS and its sulfate exhibited the effects of inhibiting DHAV infections and virus gene replications on the DEHs. Moreover, the virus inhibitory rate of sAPS was higher than those of BSRPS and sBSRPS. It accounted for the excellent anti-DHAV effects of APS and sAPS, in vitro. Nevertheless, their antioxidant capacities were not that good as comparison with those of BSRPS and its sulfate. They could not enhance the immune response of ducklings infected with DHAV, either. Thus, APS and its sulfate could not reduce or significantly reduce the mortality rates. Despite all this, both APS (at the 4th and the 8th hour) and sAPS (at all time) alleviated the hepatic pathological injury severities and reduced death numbers in the first death peak, sAPS even put off the time of the second death peak. Theoretically, APS as an antiviral drug should have treated DVH. Unlike BSRPS and sBSRPS, however, APS and sAPS exhibited anti-DHAV abilities in vitro while did not in vivo. The difference could be accounted for the theory of syndrome differentiation based on an overall analysis of the illness and the patient's condition of Traditional Chinese Veterinary Medicine. DVH is a disease of ducklings suffering from pathogenic heat and belonged to blood-heat disease. Thus, the principal treatment of DVH is removing blood-heat rather than tonifying Qi. BSRPS is a kind of heat-clearing and blood-cooling drug, and APS is a kind of Qi-tonifying drug. So, the treatments with BSRPS and sBSRPS are dialectical treatment while those with APS and sAPS are just the opposite of the wish, which is identical to the experimental results. The reaction in vivo is complicated and holistic, so Traditional Chinese Veterinary Medicine has a unique superiority as its dialectical and comprehensive view in treatment. In the prophylaxis and treatment of DVH, we hold the point that integrated traditional Chinese and Western veterinary medicine is important. And the combination is beneficial to research and develop more effective antiviral drugs against DHAV.

In conclusion, APS or sAPS could not be an important drug of treating DVH solely. Whether will be the treatment of combination of sAPS and other drugs including BSRPS and sBSRPS effective on DVH? It is worth the lucubration.

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