Treatment effect of a flavonoid prescription on duck virus hepatitis by its hepatoprotective and antioxidative ability

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ABSTRACT

Context: Duck virus hepatitis (DVH) caused by duck hepatitis A virus type 1 (DHAV-1) is an acute and lethal disease of young ducklings. However, there is still no effective drug to treat DVH.

Objective: This study assessed the curative effect on DVH of a flavonoid prescription baicalin-linarin-icaritin-notoginsenoside R1 (BLIN) as well as the hepatoprotective and antioxidative effects of BLIN.

Materials and methods: MTX method was used to test the anti-DHAV-1 ability of BLIN in vitro. We then treated ducklings by BLIN (3 mg per duckling, once a day for 5 days) to evaluate the in vivo efficacy. To study the hepatoprotective and antioxidative roles of BLIN in its curative effect on DVH, we investigated the hepatic injury evaluation biomarkers and the oxidative stress evaluation indices of the ducklings.

Results: On duck embryonic hepatocytes, DHAV-1 inhibitory rate of BLIN at 20 μg/mL was 69.3%. The survival rate of ducklings treated by BLIN was about 35.5%, which was significantly higher than that of virus control (0.0%). After the treatment of BLIN, both the hepatic injury and the oxidative stress of infected ducklings alleviated. At the same time, a significant positive correlation (p < 0.05) existed between the hepatic injury indices and the oxidative stress indices.

Conclusions: BLIN showed a significant curative effect on DVH. The antioxidative and hepatoprotective effects of BLIN made great contributions to the treatment of DVH. Furthermore, BLIN is expected to be exploited as a new drug for the clinical treatment of DVH.

Introduction

Duck virus hepatitis (DVH) is an acute and lethal disease of young ducklings characterized by rapid transmission and severe hepatitis. The World Organization for Animal Health has ranked it as an animal disease requiring mandatory reporting. Three different duck hepatitis virus (DHV) serotypes (1, 2 and 3) cause the disease, and no cross-neutralization occurs among these serotypes (Ding & Zhang 2007; Zhang et al. 2014). Of these three serotypes, DHV-1 is the most common and virulent, causing more than 80% mortality in infected ducklings within 3 weeks (Cheng et al. 2009). The infected ducklings usually experience liver necrosis, haemorrhage and opisthotonos (Liu et al. 2008).

In the virus taxonomy of the Ninth Report of the International Committee on Taxonomy of Viruses, DHV-1 was classified as a member of a novel genus Avihepatovirus Picornaviridae, and was renamed duck hepatitis A virus (DHAV) (Fu et al. 2008). DHAV genus is genetically divided into three types: DHAV type 1 (DHAV-1), DHAV type 2 and DHAV type 3. Among them, DHAV-1 is common and widely distributed one.

Hyperimmune serum and egg yolk antibody are the most common therapeutic treatments for DVH. The protection offered by the hyperimmune serum and egg yolk antibody is not impregnable due to the weak antigenicity of the virus. Even relapse cases often happen in the clinic where patients have received hyperimmune serum and egg yolk antibody. In addition, outbreaks of DVH still occur in many ducklings that have been vaccinated with DVH attenuated vaccine (Li et al. 2013). Currently, there is no effective drug to treat DVH, and the disease causes huge economic losses once clinical cases emerge. Hence, the need to develop an effective drug treatment for DVH is urgent.

In traditional Chinese veterinary medicine, DVH belongs to pestilence and is caused by exogenous pathogenic factors. The main syndromes of the DVH are the blood-heat and liver-wind agitations. The therapeutic principle for DVH, which is based on the theory of syndrome differentiation, is cleansing the heat, removing the toxicity, strengthening the immunity of the body, eliminating the evil, cooling the blood and protecting the liver. As traditional Chinese veterinary medicine pays great attention to syndrome differentiation, the use of particular Chinese herbs is required based on the specific syndromes in the course of the different diseases. The prescriptions used for treating diseases are usually composed of several to dozens of types of Chinese herbs and are therefore, complicated. Determining which types of ingredients play the main roles in the therapeutic process, or which roles they play, are difficult. The nebulous nature of these determinations is the main reason that traditional Chinese veterinary medicine cannot be understood or even be accepted by most researchers. In addition to a mixture of herbs, single Chinese herbs are also used to treat disease. The single ingredient of one
Chinese herb is simpler to use than the prescription; however, the effect is poor, especially for the complicated and comprehensive diseases or severe pestilences such as DVH. Therefore, the shortcomings of using a single herb or preparing a complicated prescription might be avoided by composing a prescription of the ingredients that are well-known. Flavonoids are important ingredients in Chinese herbs. Flavonoids possess antioxidative, antiproliferative, antitumour, antimicrobial and anti-inflammatory activities and are widely used to treat cancer, cardiovascular disease and neurodegenerative disorders (Singh et al. 2014). Moreover, flavonoids are some of the most important active ingredients, which can enhance the immune function of an organism and inhibit the viral infection (Kong et al. 2006).

Based on the theories of the Chinese veterinary medicine, huang qin (Baikal skullcap) is a hepatic protector. Baicalin, as its main ingredient, has an antiviral property (Moghaddam et al. 2014), and is reportedly used for the treatment of viral hepatitis (Kang et al. 2014). In addition, epimedium supports the liver indirectly. It is reported that its main ingredient, icariin, possessed anti-DVH ability (Xiong et al. 2014). Notoginseng flavones (notoginsenoside R1 as a main ingredient) and Indian chrysanthemum flavones (linarin as a main ingredient) significantly inhibited the cellular infectivity of the Newcastle disease virus in chicken embryo fibroblasts (Zhang et al. 2012). So, these drugs were selected for composing the flavonoid prescription.

We designed a prescription of baicalin-linarin-icariin-notoginsenoside R1 (BLIN) according to the principles of formulating prescriptions in traditional Chinese veterinary medicine. The anti-DHAV-1 activity of BLIN was confirmed by in vitro and in vivo tests. To explore the possible antiviral mechanism of the prescription, we also investigated the hepatic injury evaluation biomarkers and the oxidative stress evaluation indices of the ducklings.

**Materials and methods**

**Ethics statement**

All animal experiments in our work conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, Eighth edition, 2011) and were approved by the Nanjing Agricultural University Animal Care Committee.

**Reagents**

Dulbecco’s modified eagle medium (Gibco, Grand Island, NY) was prepared according to the instruction for use, then supplemented with penicillin 100 IU/mL, streptomycin 100 IU/mL and 10% fetal bovine serum (V/V), which was used as nutritive medium. In the maintenance medium, the foetal bovine serum concentration was reduced to 1%. The pH of the maintenance medium, nutritive medium and Dulbecco Hanks’ Balanced Salt Solution (D-Hanks’) was adjusted to 7.3–7.5 using 5.6% NaHCO₃. Trypsin (Amresco, Solon, OH) was dissolved with D-Hank’s at 2 mg/mL, and the pH was then adjusted to 7.5–8.0. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Amresco, Solon, OH) was dissolved with calcium and magnesium-free phosphate-buffered saline (CMF-PBS) into 5 mg/mL. These reagents were filter sterilized through 0.22 μm filter membranes. The maintenance medium and nutritive medium were stored at 4 °C, the MTT solution at 4 °C in dark bottles, and the trypsin at −20°C. Other chemicals used in the experiment were of analytical grade.

The alanine aminotransferase (ALT) kit, aspartate aminotransferase (AST) kit, total protein (TP) kit, globulin (GLO) kit and albumin (ALB) kit were the products of AusBio Laboratories Co., Ltd (Beijing, China). The malondialdehyde (MDA) kit, superoxide dismutase (SOD) kit, catalase (CAT) kit and glutathione peroxidase (GSH-Px) kit were bought from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu Province, China). The PrimeScript™ RT Master Mix Kit and SYBR® Premix Ex Taq™ (Tli RNaseH Plus) Kit were the products of Takara Biotechnology Co., Ltd (Otsu, Shiga, Japan).

**Preparation of the prescriptions**

The prescription was comprised of four flavonoids: baicalin (98.00%), linarin (95.00%), icariin (98.00%) and notoginsenoside R1 (93.00%) which were purchased from Nanjing Zelang Medicine Science and Technology Co., Ltd (Nanjing, Jiangsu Province, China). The ratio of baicalin:linarin:icariin:notoginsenoside R1 was 4:2:1:6:1 which was according to the theory of monarch, minister, assistant, guide (Yi et al. 2010; He et al. 2012) in traditional Chinese veterinary medicine.

For the in vitro tests, BLIN were diluted into four concentrations in twofold serial dilutions from 20 μg/mL (the concentration had no toxicity to duck embryonic hepatocytes (DEHs) which was determined by pre-experiment of cytotoxicity test) to 2.5 μg/mL with the maintenance medium that contained glycerinum (as cosolvent, 1%), pH was adjusted to 7.5 using 5.6% NaHCO₃. For the in vivo test, BLIN was diluted to 20 mg/mL (net content of the total ingredients) with distilled water and glycerinum (as cosolvent, 1%), pH was adjusted to 7.5 using 5.6% NaHCO₃.

**DEHs and DHAV-1**

The DEHs were obtained according to our previous method (Chen et al. 2014a). The nutritive medium was discarded when the DEHs had grown into a monolayer. The monolayer of the DEHs was washed with CMF-PBS once for the assay in vitro. The DHAV-1 (LO₂ strain) was supplied by the Shandong Institute of Poultry (Jinan, Shandong Province, China). The virus was diluted with maintenance medium for the in vitro assay and with physiological saline for the in vivo assays.

**Anti-DHAV-1 activities of BLIN in vitro and in vivo**

**In vitro test**

The cell control (CC) group, virus control (VC) group and prescription group were set up in the 96-well plate containing the monolayer of the DEHs. The prescription group included four concentrations and five repetitions per concentration. For treatment, 100 μL of the DHAV-1 solution (50 TCID₅₀) was added to each well in the VC and prescription groups, and an equivalent amount of the maintenance medium was added to the wells in the CC group. Then, the plate was incubated for 1 h at 37 °C in a humid atmosphere of 5% CO₂. The virus solution and maintenance medium were removed, and the monolayer of the DEHs monolayer was washed three times with D-Hank’s. Then, 100 μL dilutions of the prescription were added into the prescription treatment group. At the same time, 100 μL maintenance medium with glycerinum (as cosolvent, 1%) was added to the CC and VC groups. The plate was allowed to continue incubation at 37 °C for 72 h, and the DEHs viability was then measured by MTT.
colorimetric assay (Huang et al. 2013). The virus inhibitive rate was calculated based on the formula: virus inhibitive rate = \((A_{\text{prescription}} - A_{\text{VC}})/(A_{\text{CC}} - A_{\text{VC}}) \times 100\%\).

### In vivo test

Three-day-old Cherry Valley ducklings (Purchased from Tanguan Poultry Farm, Nanjing, Jiangsu Province, China) were injected intramuscularly with 0.2 mL DHAV-1 solution per duckling (10 LD\(_{50}\)) except for those treated as the blank control (BC) group (injected with the same volume of physiological saline, reared in isolation, 31 ducklings). The ducklings injected with the DHAV-1 were divided randomly into VC group and BLIN group, 31 ducklings per group. BLIN group were orally administered with BLIN at a dosage of 0.15 mL per duckling, once a day for 5 days. To ensure the consistency of the assay, ducklings in the BC and VC groups were treated with the same volume of the solution with 1% cosolvent. The survival rate of each group was calculated until no death was found. Following death, the ducklings were disposed of according to local standard biosafety protocols.

### Therapeutic mechanism of BLIN

#### Animal grouping and treatments

Three-day-old Cherry Valley ducklings (purchased from Tanguan Poultry Farm, Jiangsu Province, China) were randomly divided into the BC group (isolation reared), VC group and BLIN group, with 60 ducklings per group. Ducklings in the BC and VC groups were injected intramuscularly with 0.2 mL DHAV-1 were divided randomly into VC group and BLIN group, 31 ducklings per group. BLIN group were orally administered with BLIN at a dosage of 0.15 mL per duckling, once a day for 5 days. One hour later, ducklings in the BLIN group were orally administered aqueous BLIN solution. The dosage was 0.15 mL per duckling, once a day for 5 days. Ducklings in the BC and VC groups were treated with an equal amount of the solution with the added solvent for 5 days.

At the earlier (4th and 8th h) and later (54th h) stages (Chen et al. 2014b), blood samples were randomly collected from five ducklings per group. The number of these ducklings (15 ducklings per group) was not included in the sample number. Thirty microliters of blood was added into 1 mL Trizol for total RNA extraction. The serum levels of the ALT, AST, ALP, LDH, TP and ALB were measured by MDA, SOD, CAT and GSH-Px kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer’s instructions. The serum GLO content was calculated by the serum TP content minus the ALB content.

### Evaluation biomarkers for liver damage

The serum levels of the ALT, AST, ALP, LDH, TP and ALB were tested by enzymatic colorimetry using an automatic biochemical analyzer (7180 Automatic Analyzer, HITACHI, Japan) at the Nanjing Shihuang Institute of Animal Science and Technology (Nanjing, Jiangsu Province, China). The serum GLO content was calculated by the serum TP content minus the ALB content.

### Statistical analysis

The data of the A\(_{570}\) values were expressed as the mean ± S.E. Duncan’s multiple range tests were used to analyze the differences among groups with the software SPSS 20.0. The chi-square test was used to analyze the differences among the survival rates. Significant differences were considered as \(p < 0.05\).

### Results

#### Anti-DHAV-1 activities of BLIN in vitro and in vivo

Table 1 shows the anti-DHAV-1 activity of BLIN in vitro. After the treatment with BLIN at four concentrations, the A\(_{570}\) values of DEHs were all significantly higher (\(p < 0.05\)) than that of VC. And, BLIN showed a dose-dependent virus inhibitory effect. The highest DHAV-1 inhibitory rate of BLIN was 69.3% when the concentration was 20 μg/mL. When were infected by DHAV-1, all ducklings died (Table 2). After the treatment of BLIN, the survival rate of ducklings was 35.5%, which was significantly higher (\(p < 0.05\)) than that of VC group (0.0%).
The dynamic death and survival rates of each group are illustrated in Figure 1(A–B). The survival rates of the BC and BLIN groups were 100.0% and 37.8%, respectively, which were significantly higher than that of the VC group (0.0%). The dynamic death of the BLIN group was similar to that of the VC group in general: first, it increased, and then it decreased, with mass death occurring within 48 h. The death peak of the VC group was from hours 24 to the 36, whereas the death peak in the BLIN group was shorter, occurring between the 24th h and the 30th h.

Pathological change of the liver

The visual pathological changes of the livers in the three groups are illustrated in Figure 2(A–C). The liver in the BC group was a natural brown colour, soft, of uniform colour and luster, with regular shape, and no pathological changes were observed. The pathological changes in the liver of the VC group were severe, the colour was pale, and the shape indicated swelling. The surface of the liver was full of petechiae and ecchymosis, and the border of the liver had become dull. After the administration of the BLIN, the pathological changes were clearly alleviated: the petechiae were fewer than in the VC group, and no ecchymosis was visible in the liver. The H&E staining of the liver in each group is presented in Figure 2(D–F). The H&E staining showed that the livers of BC group were normal. There was much lymphocyte infiltration in the livers of the VC group, with many extravasated blood. Obviously, serious necrosis of the liver cells was also observed. In the BLIN treatment group, only some lymphocyte infiltration and extravasated blood were present.

Dynamic changes of DHAV-1 gene expressions in blood

The dynamic DHAV-1 gene expression in the blood is illustrated in Figure 3. The DHAV-1 gene was not detected in the BC group during the sampling time. The DHAV-1 gene expression at the 4th h of the VC group was set to 1. The relative expression of the DHAV-1 gene in the BLIN group was always obviously lower than that of the VC group at each sampling time.

Results of the evaluation of biomarkers of the hepatic injury

The results of the evaluation biomarkers of the hepatic injury at the earlier (the 4th and 8th h) and later (the 54th h) phases are presented in Table 3.

The activities of the serum ALT and AST of the VC group were markedly higher than those of the BC group at all sampling times. There were prominent reductions in the ALT and AST activities at the 8th and the 54th h of the BLIN group when compared with that of the VC group. The dynamic changes of the ALP and LDH were similar, and no significant differences were observed among the three groups at the earlier phase. At the later phase, the serum ALP and LDH activities of the VC group increased remarkably, whereas those of the BLIN group were maintained at the control value.

The serum TP, ALB and GLO contents of the VC group were noticeably decreased in comparison with the BC group except for the GLO content at the 4th h. The TP, ALB and GLO contents of the BLIN group maintained normal levels at the earlier phase and decreased at the later phase. Furthermore, the contents of TP, ALB and GLO of the BLIN group were markedly higher than those of the VC group.

Results of evaluation indices of oxidative stress

To determine the degree of oxidative stress, the serum MDA, SOD, CAT and GSH-Px levels at the 8th and 54th h were measured, and the results are displayed in Figure 4. No notable increase in the MDA level in the VC group was found until the 54th h. The MDA level of the BLIN group decreased noticeably when compared with that of the BC and VC groups at the 8th and the 54th h. The changing trends of the SOD, CAT and

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

| Group | Concentration ($\mu$g/mL) | $A_{570}$ | Virus inhibitory rate (%) |
|-------|--------------------------|----------|---------------------------|
| BLIN  | 20                       | 0.565 ± 0.030$^b$ | 69.3%±9.6%$^a$ |
|       | 10                       | 0.485 ± 0.024$^{bc}$ | 43.1%±7.7%$^{ab}$ |
|       | 5                        | 0.469 ± 0.038$^c$ | 37.9%±12.4%$^{ab}$ |
|       | 2.5                      | 0.453 ± 0.013$^d$ | 32.6%±4.2%$^b$ |
| CC    |                           | 0.659 ± 0.008$^d$ |                           |
| VC    |                           | 0.353 ± 0.026$^d$ |                           |

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

The concentrations had no toxicity to DEHs which was determined by pre-experiment of cytotoxicity test.

Table 2. Survival rate of ducklings after treating with BLIN.

| Group | Sample number | Survival number | Survival rate (%) |
|-------|---------------|-----------------|-------------------|
| BC    | 31            | 31              | 100.0$^a$        |
| VC    | 31            | 0               | 0.0$^c$          |
| BLIN  | 31            | 11              | 35.5$^b$         |

Data within a column without the same superscripts (a–c) differ significantly ($p < 0.05$).
GSH-Px activities were almost the same. No distinct differences were observed in the SOD, CAT and GSH-Px activities among the three groups at the 8th h except for the GSH-Px activity of the BLIN group. At the 54th h, the SOD, CAT and GSH-Px activities of the VC group decreased distinctly when compared with those of the BC and BLIN groups.

Correlation analysis between the hepatic injury and oxidative stress evaluation indices

Table 4 lists the Pearson correlation coefficients between the hepatic injury and oxidative stress evaluation indices at the 54th h. MDA showed a significant positive correlation with ALT, and remarkably negative correlations with TP, ALB and GLO. A significant negative correlation was found between SOD and ALT, AST, ALP and LDH; between CAT and AST, ALP and LDH; and between GSH-Px and ALT, ALP and LDH. A significant positive correlation existed between GSH-Px and TP, ALB and GLO.

Discussion

The MTT method was used to reflect the amount and vitality of the living cells (Scherlie 2011) and was applied to verify the anti-DHAV-1 activity of BLIN on DEHs in this study. The virus inhibition rate reflects the antiviral effect of drugs directly (Fan et al. 2011). As the results showed in Table 1, BLIN showed a significant inhibitory effect on the DHAV-1 infection. This finding is consistent with the results of a previous study (Fan et al. 2011).
fine anti-DHAV-1 ability. The in vitro environment is simple, and the antiviral activity in vitro is due only to the direct impact on the virus and/or target cells. The in vivo environment is relatively complicated. Furthermore, not only the direct antiviral ability but also the indirect effect is important for treating DVH because it is an acute and serious disease. Therefore, in vivo test is necessary for the assessment of anti-DVH effect of BLIN. The significantly increased survival rate of the BLIN group indicated that this prescription possessed therapeutic effects for DVH. Therefore, both in vitro and in vivo tests testified the anti-DHAV-1 ability of BLIN.

The survival rate of the BLIN group (37.9%, Figure 1(B)) in the following therapeutic mechanism assays was approximately the same as the preliminary in vivo test (35.5%, Table 2), which was also notably elevated when compared with that of the VC group. The assay showed that BLIN decreased the death during the death peak and shortened the duration of the death peak (Figure 1(A)), and it also prolonged the survival time of the infected ducklings. BLIN not only increased the survival rate of the ducks but also alleviated the lesions in their livers (Figure 2). The lymphocyte infiltration and blood extravasation were less in the BLIN group than in the VC group. Furthermore, no obvious necrosis of the liver cells was observed in the livers of the BLIN group.

Both the visual inspection and H&E staining demonstrated that the livers were injured badly in the VC group, and the BLIN alleviated such injury. To further quantify this conclusion, the evaluation biomarkers of the hepatic injury, which included ALT, AST, ALP, LDH, TP, ALB and GLO, were detected. The serum ALT and AST levels rose with the degree of hepatic injury (Liu et al. 2014; Vespasiani-Gentiliucci et al. 2014). At all time, ALT and AST in ducklings of the VC group were significantly higher (p < 0.05) than those of the BC group, which suggested the livers in the VC group were injured (Table 3). After oral administration of BLIN, ALT and AST levels started declining from the 8th h, which revealed that BLIN prevented the hepatocytes from being injured. The ALP and LDH activities increase when hepatic necrosis occurs in hepatitis (Mamari et al. 2013). The ALP and LDH activities of the VC group increased remarkably, whereas the ALP and LDH activities of the BLIN group remained in the control range, which implied that BLIN prevented the hepatic necrosis caused by the DVH. From above, the BLIN might have hepatocyte-protective capacity, which prevented the hepatic injury caused by the DVH.

The dynamic DHAV-1 gene expression in blood reflected the DHAV-1 content in the blood directly. The virus was injected intramuscularly into the organism, and subsequently was dispersed to the target organ through blood circulation. At the 4th h, the DHAV-1 had not completed a replication cycle as observed in normal conditions. The distinct reduction in the DHAV-1 gene expression implied that BLIN provided a direct anti-DHAV-1 function (Figure 3). At the 4th h, the TP content of the VC group decreased observably in comparison with that of the BC group (Table 3). This decrease suggested that further attacks on the DHAV-1 might lead to a greater reduction in the TP. The DHAV-1 proliferated rapidly in the target organ and released virions into the circulating blood, which led to a relative decrease in the TP.

![Figure 4](image-url)

**Figure 4.** The influence of BLIN on the oxidative stress. A, MDA; B, CAT; C, SOD; D, GSH-Px. Bars with different letters (a–c) at the same time are different significantly (p < 0.05).

|       | ALT  | AST  | ALP  | LDH  | TP   | ALB  | GLO  |
|-------|------|------|------|------|------|------|------|
| MDA   | 0.760* | 0.491 | 0.633 | 0.678 | -0.978* | -0.933* | -0.959* |
| SOD   | -0.840* | -0.941* | -0.913* | -0.874* | 0.553 | 0.727 | 0.391 |
| CAT   | -0.635 | -0.887* | -0.830* | -0.781* | 0.254 | 0.401 | 0.128 |
| GSH-Px| -0.767* | -0.668 | -0.773* | -0.784* | 0.848* | 0.873* | 0.782* |

*p < 0.05.
increase in DHAV-1 expression to 2.553 at the 8th h in the VC group. At the 8th h, no notable difference was observed in the TP and ALB contents between the VC and BLIN groups, whereas the GLO content of the BLIN group was higher than that of the VC group. GLO has an immune function. This phenomenon revealed that the ducklings in the BLIN group might mobilize the immune system to resist attack by the DHAV-1. The DHAV-1 gene expressions at the 54th h decreased sharply both in the VC and BLIN groups. This might be due to the viremia fading away over time. Even so, the DHAV-1 gene expression of the BLIN group was significantly lower than that of the VC group. All the above cases demonstrated that the BLIN inhibited the DHAV-1 gene expression.

 Diseases caused by an unfavourable environment (such as high temperature and radiation) and pathogen infection lead to oxidative stress (Schrader & Fahimi 2006). In most liver diseases, including those of metabolic and viral origin, oxidant stress acts as an important determining factor in the development of diseases (Choi 2012; Tell et al. 2013). Our previous assay (Chen et al. 2014b) also demonstrated that DHAV-1 caused the oxidative injury. In vivo, the free radicals attack unsaturated fatty acid on the biological membrane, triggering lipid oxidation. SOD, CAT and GSH-Px play key roles in scavenging free radicals (Fernandez-Checa & Kaplowitz 2005; Tell et al. 2013). The concentration of MDA, as the final product of lipid oxidation (Cerretani et al. 2011), reflects the state of lipid oxidation in vivo and the extent of oxidative stress. At the 8th h, the serum MDA, SOD, CAT and GSH-Px of the BC and VC groups were almost at the same levels, which exhibited that the oxidation and the antioxidative defences were in the dynamic balance at the earlier stage in the course of DHV. When the level of oxidation exceeds the antioxidative defences, oxidative stress occurs (Zampetaki et al. 2013). At the 54th h, the SOD, CAT and GSH-Px activities of the VC group decreased notably when compared with those of the BC group, and the MDA content of the VC group increased markedly, which demonstrated that the DHAV-1 caused the depletion of the antioxidant, accumulation of the free radicals and disruption of the free radical balance.

 The formation of free radicals is inhibited by an antioxidative system that includes nonenzymatic (i.e., vitamins C, A, and E and glutathione) and enzymatic (Garcia-Fernandez et al. 2008) antioxidants. The SOD catalyzes the superoxide radical forms into hydrogen peroxide (Mamari et al. 2013). Hydrogen peroxide, although it has no unpaired electrons, still belongs to the reactive oxygen species and does harm to the body. With the catalysis of CAT and peroxide (GSH-Px is an important one), hydrogen peroxide, as well as the other peroxides, eventually will be broken up into harmless water and hydroxyl compounds (Fernandez-Checa & Kaplowitz 2005). Following the oral administration of BLIN for 54 h, the SOD, CAT and GSH-Px activities were significantly higher than those of the VC group, and almost no obvious differences were observed with the BC group (Figure 4), which demonstrated that BLIN improved the activities of the antioxidases. Furthermore, the increased SOD, CAT and GSH-Px activities mean the ducklings in the BLIN group possessed a stronger capacity for scavenging free radicals, which alleviated the oxidative stress. As Table 4 shows, significantly negative correlations exist between the SOD, CAT and GSH-Px and the ALT, AST, ALP and LDH (except for between the CAT and ALT and GSH-Px and AST), which implies that the higher the activities of the SOD, CAT and GSH-Px, the lower the levels of the ALT, AST, ALP and LDH, that is, the lower the hepatic injury. The balance between the production and decomposition of the free radicals is very delicate, and if this balance tends to overproduce the free radicals, the cells start to suffer the consequences of oxidative stress (Carocho & Ferreira 2013).

 Hepatic injury was observed as early as 4 h after being challenged with DHAV-1, whereas the balance of free radicals was not disturbed until the later phase of the course of the DVH. This phenomenon suggested that the hepatic injury might be the reason for the disequilibrium of the free radicals. Therefore, the hepatic injury resulted from the cell damage, and the damaged hepatocytes might have caused the accumulation of the free radicals, which depleted the antioxidases and thus led to the decrease in the SOD, CAT and GSH-Px (Olsvik et al. 2005; Valko et al. 2007). The same results were obtained in our present assay (Figure 4). Depletion of the SOD, CAT and GSH-Px below a critical threshold thus favoured the accumulation of free radicals, and this in turn triggered the hepatocyte dysfunction and necrosis, which led to a damaging cycle (Begriche et al. 2011). The damage created by the oxidative stress not only affects the lipids but also affects other major cellular components including the proteins and DNA (Tell et al. 2013). A remarkably negative correlation between the MDA and the TP, ALB and GLO was found in our present work (Table 4). Due to the reasons of oxidative stress and attack by DHAV-1, the hepatic injury of the VC group was severe at the 54th h (Table 3 and Figure 2(A)).

 At the earlier stage of the infection, DHAV-1 amount increased as time went on (Figure 3). But the free radicals in the VC groups were at the normal level (Figure 4). BLIN decreased the DHAV-1 amount and alleviated the hepatic injury (Table 3) at the earlier stage. It indicated that BLIN alleviated the hepatic injury by its inhibiting virus proliferation effect at this stage. At the later stage of the infection, DHAV-1 amount was not very large (Figure 3). However, in VC groups the free radicals increased (Figure 4). In BLIN groups, the hepatic injury and the free radicals were significantly lower than that in VC groups (Table 3). It indicated that BLIN alleviated the hepatic injury by its antioxidative effect at this stage. Thus, BLIN alleviated the hepatic injury by inhibiting virus reproduction at the earlier stage and scavenging free radicals at the later stage. Eventually, the survival rate of the BLIN group was increased significantly when compared with that of the VC group.

 Modern medical research has shown that flavonoids have antiviral, antibacterial, anti-inflammation, antioxidant and cell membrane-protective activities (Singh et al. 2014). All activities listed above might be of great significance in the course of DVH. BLIN is composed of four types of flavonoids. The outstanding performance of BLIN is likely dependent on the antioxidant activity of these four flavonoids. Of course, the exact pharmacological actions of BLIN need to be further studied.

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Disclosure statement

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