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Effects of alfalfa saponin extract on the performance and cholesterol metabolism of laying hens

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Abstract

The experiment was performed to determine the effects of alfalfa saponin extract (ASE) on the performance and cholesterol metabolism of laying hens. A total of 150 Hy-Line Brown hens with 28 weeks old, were randomly divided into five treatment groups (five replicates per treatment with six hens per replicate). Diets containing 0, 60, 120, 240, and 480 mg ASE/kg were fed to hens for 77 days. The shell thickness had a trend to increase. The yolk cholesterol and liver bile acid decreased significantly (ASE 60 and 480 mg/kg groups for yolk cholesterol, and ASE 60 and 240 mg/kg groups for liver bile acid). Fecal bile acid has an elevation trend as ASE increased. The expression of very low density apolipoprotein-II (*apoVLDL-II*) gene was not affected by adding ASE. However, the mRNA expression of 3-hydroxy-3-methylglutaryl coenzyme A (*HMG-CoA*) reductase gene and cholesterol 7α-hydroxylase (*CYP7A1*) gene were significantly up-regulated. The mRNA expression of very-low-density-lipoprotein receptor (*VLDLR*) gene was suppressed due to adding ASE supplementation in the diet. These findings indicated that dietary ASE could regulate cholesterol levels in hens by up-regulating the mRNA levels of *HMG-CoA* and *CYP7A1* and suppressing the expression of *VLDLR*.

Key Words: Alfalfa saponin extract, Chicken, Cholesterol metabolism, mRNA expression

1. Introduction

With the increasing concerns over food safety, environment contamination, and general health risks, the search for decreasing the high fat content of animal products is indeed needed. Alfalfa is very effective in decreasing egg cholesterol with the least loss in egg production, egg size, and feed efficiency (Menge *et al*., 1974; Shi *et al*., 2014). Previous studies suggested that alfalfa saponins might prevent hypercholesterolemia, reduce egg production, and suppress growth in mammals and birds (Anderson, 1957; Heywang and Bird, 1954; Heywang *et al*., 1959; Malinow *et al*., 1981; Malinow *et al*., 1977; Malinow *et al*., 1979; Malinow *et al*., 1980; Whitehead *et al*., 1981). Aqueous alfalfa extract also have been reported to alter the lipid metabolism of chicken (Deng *et al*., 2012; Dong *et al*., 2007).

Cholesterol metabolism in laying hens has been under investigation for several decades. Cholesterol intake from a conventional laying diets could not meet the hens' needs, requiring

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hens to synthesis most of the cholesterol needed for the egg, as structural components of cell membranes, and as precursors to sex and adrenal hormones, vitamin D, and the bile acids (Hargis, 1988). Cells must balance the internal and external sources of cholesterol so as to maintain mevalonate biosynthesis while at the same time avoiding the accumulation of excessive cholesterol, which can result in diseases such as atherosclerosis, gallstones and several lipid storage disorders (Goldstein and Brown, 1990). It is well established that cholesterol homeostasis is maintained in normal animals through two regulated pathways: *de novo* synthesis and catabolism (Peet *et al*., 1998). HMG-CoA reductase and CYP7A1 are rate-limiting enzymes of cholesterol synthesis. VLDLR and apoVLDL-II are largely determined the amount of cholesterol transported into the egg yolk. ASE has been associated with alterations on lipid metabolism, most probably by binding to dietary lipids in intestin and thus decreasing their absorption and increasing their excretion (Francis *et al*., 2002). However, how ASE affects the lipid metabolism, especially the gene expression of cholesterol metabolism is still remained unknown.

The objective of this study was to further describe the effect of dietary ASE on the performance, serum lipid indices and the gene expression of cholesterol metabolism.

2. Materials and methods

2.1 Animals and diets

Animal experiments and procedures were approved by the Institutional Animal Ethics Committee of Henan Agricultural University (Permit Number: 11-0085). All efforts were made to minimize the suffering of the animals. A total of 150 healthy Hy-Line Brown hens at the age of 27 weeks were randomly distributed into five treatment groups (with five replicates per treatment and each replicate consists of six hens). The hens were reared in wired cage (48 × 38 × 34 cm^3) with two hens per cage. Feed and water were supplied *ad libitum* throughout the 77-day experimental period under standard management conditions (Zhou *et al*., 2014). A regime photoperiod (16-h light/8-h dark) was provided for all birds. All hens were staving healthy during the experimental period. The ingredients and nutrient level of basic diet were shown in Table 1. All diets contained adequate levels of nutrients according to the National Research Council's recommendation (NRC, 1994).

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Experimental diets contained 0, 60, 120, 240, or 480 mg ASE/kg was assigned to groups CK, A, B, C, and D, respectively. ASE was provided by Hebei Bao'en Biotechnology Co., Ltd (Shijiazhuang, China) which contained 61.64% saponins, 10.97% flavonoids, 8.12% polysaccharides, 7.11% moisture, and 12.16% unknown compounds as described (Zhou *et al*., 2014).

2.2 Sampling and measurements

Before the formal experiment, there was a 7-day pre-feed period. Eggs from each replicate were collected and weighed daily at the same time point to get the daily egg production, egg weight, and egg mass. Feed consumption was recorded weekly and calculated per day for each hen. Twenty eggs were randomly collected from each treatment group on the last day of every two weeks to assess egg quality, as well as yolk cholesterol. The egg quality analysis included egg shape index, egg shell force, shell thickness, Haugh unit, and yolk color. Shape index (%) = [egg length (cm)/egg width (cm)] ×100. Egg quality was measured through the multifunctional automatic egg characteristics analyzer (Robotmation, Tokyo, Japan). After characterization of egg quality, yolks were subjected to homogenization then stored at -20 °C until further analysis.

At the end of the experimental period, one bird per replicate was randomly selected and weighted 12 hours after feed deprivation. Feces were collected for bile acid (BA) content analysis using total feces collection method. Fresh blood was obtained by cardiac puncture using sterilized syringes and needles. Serum samples were isolated by centrifugation at 3000 × g for 10 min at 4 °C and stored at -80 °C until further analysis. Serum traits, which includes triglycerides (TG), total cholesterol (TC), high density lipoproteins cholesterol (HDL-C), low density lipoproteins cholesterol (LDL-C), and very low density lipoproteins cholesterol (VLDL-C), were measured by automatic clinical chemistry analyzer (Hitachi, Tokyo, Japan).

After blood sampling, the birds were euthanized by cervical dislocation immediately. The liver was dissected, weighted and then homogenized before freezing for storage at-20 °C. The ovarian tissue was also dissected and marked then freezing for storage at -80 °C. The yolk cholesterol was measured through High Liquid Chromatography with methanol as a liquid carrier. And the bile acid contents of liver and feces were measured using commercially available reagent kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Total RNA was isolated using the TRIzol reagent according to the manufacture's recommendations (Invitrogen, Carlsbad, USA). RNA was assessed for quality and quantity using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, USA). Reverse transcription reaction was performed using the Reverse Transcriptase M-MLV (Rnase H^-) kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. Real-time PCR was conducted in a 20 μL reaction system using SYBR® *Premix Ex Taq*™ (Tli RNaseH Plus) on an Eppendorf Mastercycler ep realplex 2 (Eppendorf, Hamburg, Germany) following the recommendations. The primer sequences were shown in Table 2. Real-time PCR reactions were performed at 95 °C for 2 min, followed by 40 cycles at 95 °C for 20 s, 60 °C for 20 s and 72 °C for 20 s. The fluorescence signals were measured once at the end of each cycle. Dissociation analysis of amplification products was performed at the end of each real-time PCR to confirm that only one PCR product had been amplified and detected. Relative gene expression was normalized to glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.3 Statistical analysis

Data are presented as mean ± standard deviation (SD). The data were analyzed by one-way ANOVA using SPSS 22 software (SPSS, Chicago, USA). The differences between the means were determined using Duncan's test. Differences were considered significant at the level of $P < 0.05$ and $P < 0.01$ were considered very significant differences.

3. Results

3.1 Performance of laying hens

The effects of ASE on performance of laying hens are shown in Fig. 1. There was no significant difference on egg weight with ASE at 0 and 60 mg/kg ($P > 0.05$). Egg weight changed significantly with ASE of 120, 240 and 480 mg/kg ($P < 0.05$, $P < 0.01$ and $P < 0.01$, respectively) (Fig. 1A). And ASE 60, 120 and 240 mg/kg were significantly different from one another ($P < 0.01$). As shown in Fig. 1B, there were no significant differences on egg production with ASE at 0, 60, 120 and 240 mg/kg ($P > 0.05$), but the egg production decreased significantly with ASE of 480 mg/kg ($P < 0.05$). And ASE 480 was very significantly different from ASE 60, 120 and 240 mg/kg groups ($P < 0.01$). According to Fig.

1C, compared to the control group (with ASE of 0 mg/kg), feed intake decreased in all of the four treatment groups with ASE supplemented in diets. Especially with the ASE of 120 and 240 mg/kg, feed intake decreased very significantly ($P < 0.01$). There was significant difference between ASE at 0 and 60 mg/kg ($P < 0.05$), while there was no significant difference detected between ASE at 0 and 480 mg/kg ($P > 0.05$). Fig. 1D showed that there were no significant differences on feed efficiency with ASE at 0, 60, 240 and 480 mg/kg ($P > 0.05$), but a significant difference existed for ASE at 0 and 120 mg/kg ($P < 0.05$).

As summarized in Fig. 2A-C, there were no significant differences on egg shape index, egg shell strength and egg shell thickness with any one of the ASE groups ($P > 0.05$). The yolk color value (Fig. 2D) showed that there was no significant difference with ASE at 0 and 60 mg/kg ($P > 0.05$). The yolk color value decreased significantly with ASE of 120, 240 and 480 mg/kg ($P < 0.01$, $P < 0.05$ and $P < 0.05$, respectively). But there were no significant differences within the four ASE treatment groups ($P > 0.05$). Fig. 2E showed that only ASE 240 and 480 mg/kg groups differed significantly from each other ($P < 0.05$).

3.2 Cholesterol concentration and serum lipid indices

The effects of ASE on cholesterol and BA are presented in Fig. 3. The yolk cholesterol and liver bile acid decreased in all of the four ASE treatment groups compared to ASE 0 mg/kg group. Yolk cholesterol decreased significantly in ASE 60 and 480 mg/kg ($P < 0.05$). Liver BA in ASE 60 and 240 mg/kg decreased significantly ($P < 0.05$ and $P < 0.01$ separately) compared to ASE 0 mg/kg group. Liver cholesterol level elevated in ASE 60, 120 and 480 mg/kg groups, but ASE 240 group decreased. ASE 240 group showed significant difference with ASE 0, 60 and 120 mg/kg groups ($P < 0.05$), while ASE 480 group had very significant differences with either one of other four groups ($P < 0.01$). Although there were fluctuations regarding to the fecal BA in ASE groups, there were no significant differences detected ($P > 0.05$).

As shown in Fig. 4, there were no significant differences on the TC, TG, HDL-C, LDL-C and VLDL-C levels with different ASE supplements ($P > 0.05$).

3.3 Gene expression in the liver and ovary

The mRNA levels of gene *apoVLDL-II* in the liver was significantly higher in the ASE 60 mg/kg group than the ASE 0 mg/kg group through supplemental ASE in the diet of laying

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hens. However, the other treatments did not show significant change compared to the ASE 0 mg/kg group (Fig. 5A).

The mRNA levels of *VLDLR* in ovary was significantly ($P < 0.05$) suppressed by supplemental ASE in the diet ($P < 0.05$) (Fig. 5B). While the expression of *CYP7A1* (Fig. 5C) and *HMG-CoA* reductase (Fig. 5D) genes in liver had the same trend, the treatment groups with ASE 60, 120 and 240 mg/kg were significantly up-regulated compared to the groups ASE 0 and 480 mg/kg.

4. Discussion

In our study, dietary ASE had no significant effects on the performance of laying hens. Dong *et al*. (2007) showed that aqueous alfalfa extract did not exert any significant effect on the performance of broiler chickens. Deng *et al*. (2012) also reported that aqueous alfalfa extract had no significant effect on the production performance of laying hens.

Supplemented with ASE in the diet of laying hens in our experimental period, feed intake declined compared to the control group. This might because the poor palatability of ASE due to the bitterness of alfalfa saponin (Zhou *et al*., 2014). This is in accordance with the dietary saponin in laying hens (Sim *et al*., 1984). Over the experimental period, ASE supplementation had a trend to increase egg shell thickness. Kocaoğlu Güçlü *et al*. (2004) reported that alfalfa meal could significantly enhance the egg shell thickness in quails. It might be attributable to favorable alternations in the intestinal environment and function which may have increased intestinal calcium absorption. But the detailed mechanism of how ASE increases egg shell thickness remains unclear.

The results of this study indicated that ASE could significantly decrease the concentration of bile acid in the liver and promote the bile acid excreting into feces. Malinow *et al*. (1981) demonstrated that alfalfa saponins could decrease intestinal absorption of cholesterol and increase fecal excretion of endogenous and exogenous neutral steroids and bile acids. Oakenfull *et al*. (1979) also reported that commercial saponins could increase both the liver bile acid secretion and the fecal excretion of bile acids in the rat. Liver is an important organ for the cholesterol metabolism. Accumulation of cholesterol in the liver benefits cholesterol transformation into bile acid, thus speeding up the excretion of

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cholesterol. The possible mechanism is that ASE involved in bile acid enterohepatic circulation. Kocaoğlu Güçlü *et al*. (2004) found that alfalfa saponin could prevent the reabsorption of bile acids, these bile acids are diverted from enterohepatic cycle and their excretion with feces is increased.

The cholesterol of serum mainly included body biosynthesis and intestinal absorption, both of them maintained the relative balance. In this present study, ASE had no significant effects on the serum lipid indices in laying hens. This might due to the balance of cholesterol metabolism maintained by the body.

Cholesterol metabolism in all tissues of the body except the central nervous system is in a dynamic equilibrium (Gould *et al*., 1953). Cells must balance the internal and external sources of cholesterol so as to maintain mevalonate biosynthesis while at the same time avoiding the accumulation of excess cholesterol, which can result in diseases such as atherosclerosis, gallstones, and several lipid storage disorders (Goldstein and Brown, 1990). HMG-CoA reductase is a rate-limiting enzymes of cholesterol synthesis in liver (Brown and Goldstein, 1986). We found that by supplementing different levels of ASE in the diet up-regulated the mRNA levels of *HMG-CoA reductase* gene in the liver, which would promote the synthesis of cholesterol. However, significant changes in liver cholesterol level were not found. At the same time, we found that the concentration of liver bile acid was significantly decreased and the fecal bile acid was significantly increased. This showed that excess cholesterol was converted to bile acid and then excreted in feces. CYP7A1 is a rate-limiting enzyme in the classical bile acid synthesis pathway (Lehmann *et al*., 1997). In this study, the mRNA level of *CYP7A1* gene had been up-regulated by ASE. These results were consistent with the effects of bile acid excretion in feces. Previous study showed that bile acid converted from hepatic cholesterol could promote the reduction of cholesterol thus inducing compensatory in both hepatic and intestinal HMG-CoA reductase activity (Harwood *et al*., 1993). Naber *et al*. (1982) reported that another route of cholesterol excretion in the hens is provided by the egg excretion in the feces. Griffin (1992) indicated that the cholesterol content of the yolk was primarily dependent on the cholesterol content of triglyceride-rich lipoproteins. Griffin also claimed that over 95% of yolk cholesterol was associated with yolk triglyceride-rich lipoproteins (Griffin, 1992). Schneider *et al*. (1990) demonstrated that

241 triglyceride-rich lipoproteins were transported from the liver primarily to growing oocytes by
242 VLDL and concluded that the presence of *apoVLDL-II* on the laying hens VLDL ensures
243 efficient delivery of cholesterol to the oocyte for subsequent use as energy source by the
244 embryo. VLDL particles are the most predominant lipoprotein in serum transporting lipids
245 delivered to oocytes where it is bound and transported into growing oocytes via
246 receptor–mediated endocytosis (Elkin and Rogler, 1990). Schneider *et al*. (1990) indicated
247 that *apoVLDL-II* was in assurance of lipid transportation into the growing oocyte and kept
248 VLDL from transforming to other lipoprotein which could not be degraded. Chicken *VLDLR*,
249 also was called oocyte vitellogenesis receptor (*OVR*) or vitellogenin receptor (*VTGR*),
250 mediates the absorption of yolk protein precursors from plasma very low density lipoprotein
251 and vitellogenin (Wang *et al*., 2011). So the expression of *VLDLR* and *apoVLDL-II* were
252 largely determined by the amount of cholesterol transported into yolk. In our research, we
253 found that supplemental ASE had no significant effects on the mRNA level of *apoVLDL-II*
254 compared to CK. However, it significantly decreased the mRNA expression of *VLDLR*. This
255 would decrease the amount of cholesterol transport from serum into yolk. These results were
256 consistent with the significantly descent of yolk cholesterol.

257 In summary, dietary ASE is able to improve egg shell thickness and promote the
258 excretion of bile acid of laying hens. Our data also indicate dietary ASE may modulate the
259 cholesterol content by up-regulating the mRNA levels of *HMG-CoA reductase* and *CYP7A1*
260 and suppressing the expression of *VLDLR* of laying hens.

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262 **Acknowledgement:** The authors thankfully acknowledge the financial support for this
263 research by the Earmarked Fund for Modern Agro-industry Technology Research System
264 (CARS-35).

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Tables

Table 1 Ingredient composition and nutrient content of the basal diets

Item	Content
Ingredients (%; as-fed basis)	
Corn	69.80
Soybean meal	14.45
Limestone powder	8.50
Peruvian fishmeal	4.96
Calcium hydrogen phosphate	1.00
Premix*	1.00
Salt	0.20
Methionine	0.09
Nutrient composition (dry matter)	
Metabolizable energy (MJ/kg)	11.51
Crude protein (%)	16.00
Calcium (%)	3.44
Phosphorus (%)	0.59
Available phosphorus (%)	0.42
NaCl (%)	0.33
Lysine (%)	0.82
Methionine (%)	0.38
Methionine + cysteine (%)	0.63

* Premix supplied per kilogram of diet: Fe, 60 mg; Mn, 40 mg; Cu, 4 mg; Zn, 70 mg; I, 0.8 mg; Se, 0.3 mg; vitamin A, 9000 IU; vitamin D, 2000 IU; vitamin E, 15 IU; vitamin K, 2 mg; vitamin B_{12}, 1 mg; biotin, 0.30 mg; choline chloride, 250 mg; folic acid, 0.75 mg; niacin, 20 mg; pantothenic acid, 5.3 mg; pyridoxine, 7.5 mg; riboflavin, 7.5 mg; and thiamin, 2.1 mg.

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371 **Table 2 Primers sequences used for gene expression**

Primer	Sequence (5' - 3')	Accession number	Product size (bp)
GAPDH			
Forward	GAACATCATCCCAGCGTCCA	NM 204305	141
Reverse	ACGGCAGGTCAGGTCAACAA		
VLDLR			
Forward	CGTCTGTATTGGCTTGATTCT	NM 2052129	173
Reverse	GCACCATAGACTGCCTCGTT		
HMGR[*]			
Forward	TAGAGATAGGGACTGTTGGAG	NM 204485	141
Reverse	TCACTGTAGCACACACGATT		
apoVLDL-II			
Forward	TGGTCAGTTCTTGCTGGATGTT	NM 205483	98
Reverse	GTTCCGCCAGTTTAGTGAGTCT		
CYP7A1			
Forward	CCGAGTTGCTAAGGAGGATT	NM 0010011753	191
Reverse	CGTTGCGGTAGAAGTCAGTC		

372 [*] *HMGR – HMG-CoA reductase*.