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Effect of Ultra-fine Traditional Chinese Medicine Compounds on Regulation of Lipid Metabolism and Reduction in Egg Cholesterol of Laying Hens

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■Keywords

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ABSTRACT

This study has the objective of investigating the effects of traditional Chinese medicine prescriptions (TCM) on serum lipid, abdominal and hepatic fat percentage, cholesterol content in eggs, and mRNA expression of genes apoA I and $apoB_{100}$. One hundred and thirty five healthy (300-day-old) layers were randomly assigned to three treatments. The hens in control group were fed with the basal diet. The hens in the experimental groups (TCM 1 and TCM 2) were fed with the basal diet supplemented with 1% TCM 1 and 1% TCM 2 respectively over a period of 60 days. Laying performance and the serum parameters relevant to fat metabolism were measured. The results showed that no significant differences were found in average daily feed intake and egg weight among three treatments. Average daily laying rate in TCM treatments was increased, and the cholesterol content in eggs was decreased. The serum triglyceride (TG), total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels in experimental treatments were decreased ($p<0.05$), while the serum high-density lipoprotein cholesterol (HDL-C) level was increased ($p<0.05$) compared to the control group. Additionally, abdominal fat percentage decreased in TCM 1 treatment ($p<0.05$), and hepatic fat percentage decreased in both TCM treatments ($p<0.05$). The expression of apolipoproteinA I (apoA I) and $apolipoproteinB_{100}$ ($apoB_{100}$) mRNA in the liver increased in both TCM treatments ($p<0.05$). These results suggest that the diet supplemented with TCM could increase the expression of apoA I and $apoB_{100}$ mRNA in the liver, and decrease lipid content in the serum, and reduce egg cholesterol in layers.

INTRODUCTION

Lipid is one of the necessary nutrients for both human beings and animals. It is not only an energy source, but also the provider of essential fatty acids. The excessive accumulation of fats in the liver is believed to be the cause of fatty liver hemorrhagic syndrome (FLHS) in laying hens, resulting in laying rate decreased sharply, even an increase in mortality, which brings economic loss and threaten the animal welfare (Gharaghani *et al.*, 2015; Trott *et al.*, 2014; Teck *et al.*, 2011). Besides, cholesterol content in eggs is considered as a significant factor to limit their consumption although its nutritional value is very high (Patrícia *et al.*, 2013; Spence *et al.*, 2012). The evidences above suggested that the reduction of serum lipid level of layers and cholesterol content in eggs is critical for practical production cost and animal welfare. In addition, apolipoproteinA I (apoA I) and apolipoproteinB100 ($apoB_{100,}$ the main form in poultry) were reported to be associated with lipid metabolism. ApoA I and $apoB_{100}$ are the major apolipoprotein synthesized in the liver (Kristina *et al.*, 1990). ApoA I, one important protein of high density lipoproteins (HDL), participates in the lipid translocation from peripheral tissues to the liver for catabolism (Mooradian *et al.*, 2014; Sontag *et al.*, 012). ApoB$_{100}$ plays a major role in low density lipoproteins (LDL) formation, which is regarded as a ligand for the LDL receptor-mediated uptake by different tissues (Srivastava *et al.*, 2000). Previous studies showed that apoA I and apoB$_{100}$ play important roles in physiological functions on lipid metabolism (Dixon *et al.*, 1993; Srivastava *et al.*, 2000).

In recent years, along with the development of green healthy breeding, Chinese herbal medicines have subsequently been receiving more and more attention as feed additive (Liang *et al.*, 2013; Nie *et al.*, 2015). However, the effects of conventional Chinese herbal medicine, especially coarse powder, were reduced by its low content of active ingredient and low bioavailability. The technology of ultrafine pulverization was applied to enhance the dissolution rate of effective components in trials (Cho *et al.*, 2007). According to the prescription-composing principles that Chinese herbal medicine compounds have an almost infinite ability to synthesize compounds that have diverse bioactive properties (Song *et al.*, 2014).

In this study, the experimental diets supplemented with two TCM compounds were applied on layers and the effects of TCM on the regulation of lipid metabolism and cholesterol content in eggs were evaluated, in addition, it is also an attempt to explore a useful feed additive to help cover the economic loss and improve animal welfare.

MATERIALS AND METHODS

Experimental Animals

All the procedures performed on the animals were approved by the Institutional Animal Care and Use Committee for medical science of Jiangxi Agricultural University, Jiangxi, China.

Experimental Design

One hundred and thirty five Hyline layers, aged 300 days, were caged in individual slant-back cages and allowed 7 days of accommodation on the basal diet. The basal diet was formulated to meet all nutrient requirements for laying hens (NRC, 1998) (Table 1). The layers were randomly assigned to three treatment groups. Each group consisted of 45 layers (3 or 4 layers in a cage). The control group was fed with a basal diet. Meanwhile, the layers in experimental group 1 and 2 were fed with a basal diet supplemented with 1% TCM 1 and 1% TCM 2 respectively. The experiment lasted 60 days. The diets and water were provided *ad libitum* throughout this experiment. Light cycle was kept 16 h per day.

Table 1 – Ingredient and nutrient levels of basal diet (air dry basis).

Ingredients	Content(%)	Nutrient levels	Content
Corn	64.20	ME/(MJ/kg) [2)]	11.30
Soybean meal	21.00	CP(%)	17.20
Limestone	8.10	Ca(%)	3.43
CaHPO4	1.20	TP(%)	0.63
Soybean oil	4.00	AP(%)	0.45
Salt	0.31	Lys (%)	0.92
Premix[1)]	1.00	Met (%)	0.38
Lys	0.12		
DL-Met	0.07		
Total	100		

[1)] The premix provides following per kg diet: VA 8 100 IU; VD$_3$ 1 620 IU; VE 0.30 IU; VK$_3$ 0.90 mg; VB$_1$ 0.45 mg; VB$_2$ 2.70 mg; VB$_{12}$ 0.06 g; Nicotinic acid 5.70 mg; Folic acid 0.15 mg; Biotin 0.045 mg; Fe 31.30 mg; Cu 4.59mg; Mn 41.21 mg; Zn 42.04 mg; I 0.61 mg; Se 0.15 mg; choline 448.65 mg; Rice polishings 3489.53 mg.

[2)] ME is calculated value, Other nutrient levels are measured values.

Preparation of TCM

All raw materials for TCM were purchased from the Chinese medicine market of Zhangshu (Jiangxi, China). TCM 1 was composed of 6 types of dried Chinese herbs and TCM 2 was composed of 4 types of herbs (Table 2). All dried Chinese herbs were smashed through a 10 μm screen sieve, and then TCM 1 and TCM 2 were incorporated into the basal diet at the same dose of 10 g/kg, respectively.

Table 2 – Composition and content of TCM1 and TCM2 (air dry basis)

Latin name	Used part	Content (g)
TCM1		
Astragalus membranaceus	Dried rhizome	17.14
Codonopsis pilosula	Dried root	11.43
Angelica sinensis	Dried root	14.29
Crataegus pinnatifida	Dried fuit	28.57
Rhizome Atractylodis macrocephalae	Dried rhizome	11.43
Atractylodes lancea	Dried rhizome	17.14
TCM2		
Flos carthami	Dried flower	10.00
Crataegus pinnatifida	Dried fruit	50.00
Poria	Dried sclerotium	20.00
Lotus leaf	Dried leaf	20.00

TCM, traditional Chinese medicine prescriptions; TCM 1, traditional Chinese medicine prescriptions 1;

TCM 2, traditional Chinese medicine prescriptions 2. [1]Used part of TCM 1 and TCM 2 come from

Chinese pharmacopoeia (2005).

Sample Collection

Egg production, feed intake, and egg weights in each group were recorded daily. Forty-five eggs were randomly collected from each group to determine cholesterol content in the eggs in the end of the experiment. On day 1, 30, and 60, 15 hens were chosen randomly. Blood samples were taken from the brachial veins, centrifuged at 2,000g at 4°C for 10 min and stored at -20°C for further measurement. The livers were carefully removed from the body and washed in deionized water. 200 mg of tissues were excised aseptically per layers and stored in liquid nitrogen. In addition, abdominal fats were stripped and its quality was tested, and the rest of the livers were saved separately in labeled sterile plastic sampling bags

Serum Lipid Indicators analysis

The levels of serum triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were determined by using commercial available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), and the procedure was followed with the protocol from the manufacturer.

Fat Deposition and Cholesterol Content in Eggs Analysis

Crude fat was measured using soxhlet extraction method (Guo *et al.*, 2010). The cholesterol content in the eggs was determined via sulfur phosphorus iron reagent spectrophotometry (Chen *et al.*, 2008).

Real-time Polymerase Chain Reaction (RT-PCR)

Hepatic total RNA was extracted using RNAiso Plus reagent, and total RNA concentrations were determined by Nano Drop ND 2000 spectrophotometry (Thermo Scientific, Wilmington, DE). The primers of the genes apoA I, apoB$_{100}$ and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of chicken were designed based on the sequences which were obtained from the Genbank (Table 3). The purified RNA was reverse transcribed into cDNA using a Takara RT-PCR Kit (Dalian, China) and the mRNA expression level of apoA I was quantified using the Premix Ex Taq^{TM} (Probe qPCR) master mix and apoB 100 mRNA expression was quantified using the SYBR Premix Ex Taq™ II (Tli RNaseH Plus) master mix. The RT-PCR was conducted using the ABI 7500 real-time PCR machine (Applied Biosystems, Beijing, China). The program used for the amplification of the genes consisted of a denaturation step at 95 °C for 30 s, followed by 40 cycles of 94 °C for 10 s, 60 °C for 30 s, and extension at 72 °C for 30 s. The internal reference (GAPDH) gene was used as an internal control for the normalization of all the values. ΔCt(sample)=Ct(target gene)-Ct(reference gene); ΔCt(calibrator)=Ct(target gene)-Ct(reference gene); ΔΔCt=ΔCt(sample)-ΔCt(calibrator), $2^{-\Delta\Delta CT}$ was used to calculate the expression levels of genes.

Statistical Analysis

Date was analyzed using SPSS 17.0 (Chicago, IL, USA), and presented as mean ± standard error, with $p<0.05$ as criteria for significance, and $p<0.01$ for highly significant. A significant value was obtained by one-way ANOVA, Duncan's method for multiple comparisons, and least significant difference method (LSD).

RESULTS

Effects of TCM on Laying Performance

Compared to the control group, the average of daily laying rate in TCM 1 and TCM 2 treatments was increased by 6.51% ($p<0.01$) and 3.67% ($p<0.05$) respectively. Cholesterol content in the eggs in TCM 1 and TCM 2 treatments decreased by 22.19% ($p<0.01$) and 14.54% ($p<0.05$) respectively. There was no significant difference ($p>0.05$) in the average of daily feed intake and the average egg weight among the three groups (Table 4).

Table 3 – Prime and Probe sequence.

Interest genes	Reference sequence NO.	Primer sequences (5'-3')	Products Length/ bp	Annealing temperature
ApoA I	NC-006111.3	F: GGCCAGCGGCAAGGAT R: ACTCAGCGTGTCCAGGTTGTC Probe: CATCGCCCAGTTCGAGTCCTCTGC	94bp	60°C
ApoB$_{100}$	NC-006090.3	F: CCT GCC ATG GGA AAC ATT AC R: TGC AGT GCA TCA ATG ACA GA	150bp	60°C
GAPDH	NC-006088.3	F: GGTGCTAAGCGTGTTATCATCTCA R: CATGGTTGACACCCATCACAA Probe: CTCCCTCAGCTGATGCCCCCATG	70bp	60°C

Table 4 – Effects of TCM on laying performance of layers

Item	control group	TCM 1 group	TCM 2 group
Average daily laying rate, %	80.82±0.23^{aA}	86.08±0.62^{bB}	83.74±0.40^{cAB}
Average daily feed intake, g d^{-1}	112.57±1.66^{a}	112.58±1.43^{a}	112.69±2.16^{a}
Average egg weight, g	61.82±1.15^{a}	60.21±0.72^{a}	60.88±1.28^{a}
cholesterol content in eggs, mg 100g^{-1}	749.21±48.67^{aA}	582.94±22.00^{bB}	640.29±65.08^{bAB}

TCM, traditional Chinese medicine prescriptions; TCM 1, traditional Chinese medicine prescriptions 1;
TCM 2, traditional Chinese medicine prescriptions 2.
Data are mean ± standard error. (n=45, 3 replicates per treatment, 15 layers per replicate).
$^{a-c}$ within a row, means without a common lowercase superscripts are different at $p<0.05$.
$^{A-B}$ within a row, means without a common uppercase superscripts are different at $p<0.01$.

Effects of TCM on Serum Lipid

Compared to the control group, serum TG levels (Figure 1) in TCM 1 group on the 30th and 60th days decreased by 21.69% ($p<0.01$) and 20.67% ($p<0.05$) respectively, while the dietary supplementation with TCM 2 on the 30th and 60th days declined by 14.71% ($p<0.05$) and 10.7% ($p>0.05$), respectively. Serum TC levels (Figure 2) of TCM 1 and TCM 2 group decreased by 13.3% ($p<0.05$) and 11.3% ($p<0.05$) respectively on the 60th day, but no differences were recorded on the 30th day. Compared to the control group, serum LDL-C levels (Figure 3) in TCM 1 group on the 30th and 60th days reduced by 23.53% ($p<0.01$) and 15.6% ($p<0.05$), respectively. And in the dietary supplementation with TCM 2 decreased by 12.75% ($p>0.05$) and 14.68% ($p<0.05$) respectively. The HDL-C levels (Figure 4) in TCM I group on days 30 and 60 increased by 18.68% ($p<0.05$) and 22.77% ($p<0.05$) respectively, while the TG levels in TCM II group on days 30 and 60 decreased by 8.79% ($p>0.05$) and 18.81% ($p<0.05$).

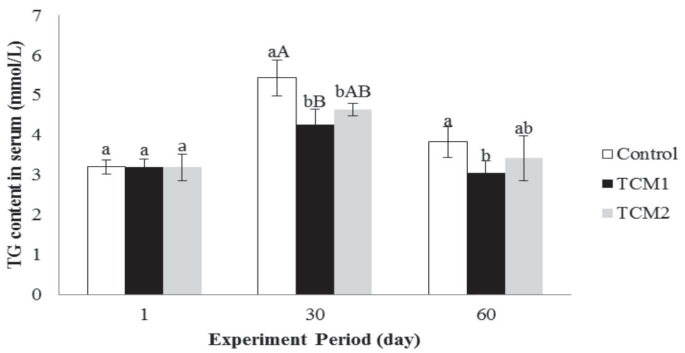

Figure 1 – TG content in serum. ^{a,b,c} $p<0.05$. $^{A, B, C}$ $p<0.01$. TCM, traditional Chinese medicine prescriptions; Control, fed with a basal diet; TCM 1, fed with a basal diet supplemented with 1% traditional Chinese medicine prescriptions 1; TCM 2, fed with a basal diet supplemented with 1% traditional Chinese medicine prescriptions 2.

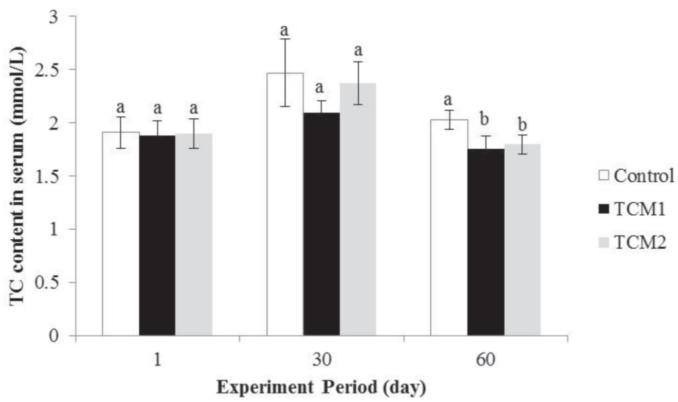

Figure 2 – TC content in serum. ^{a,b,c} $p<0.05$. $^{A, B, C}$ $p<0.01$. TCM, traditional Chinese medicine prescriptions; Control, fed with a basal diet; TCM 1, fed with a basal diet supplemented with 1% traditional Chinese medicine prescriptions 1; TCM 2, fed with a basal diet supplemented with 1% traditional Chinese medicine prescriptions 2.

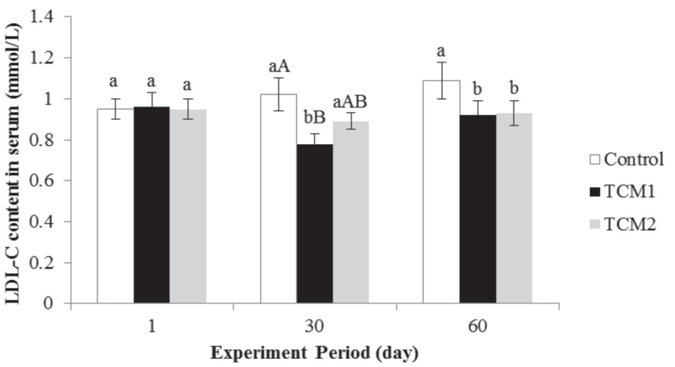

Figure 3 – LDL-CHOL content in serum. ^{a,b,c} $p<0.05$. $^{A, B, C}$ $p<0.01$. TCM, traditional Chinese medicine prescriptions; Control, fed with a basal diet; TCM 1, fed with a basal diet supplemented with 1% traditional Chinese medicine prescriptions 1; TCM 2, fed with a basal diet supplemented with 1% traditional Chinese medicine prescriptions 2.

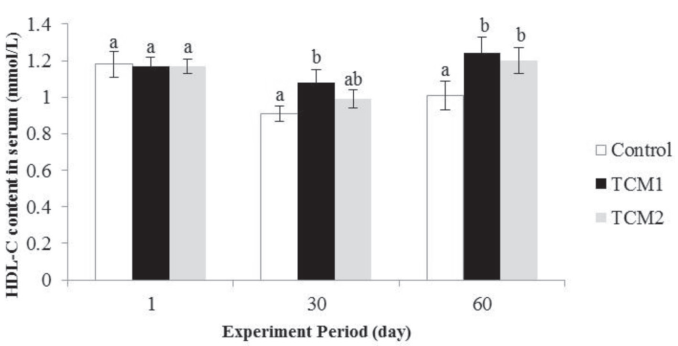

Figure 4 – HDL-CHOL content in serum. ^{a,b,c} $p<0.05$. $^{A, B, C}$ $p<0.01$. TCM, traditional Chinese medicine prescriptions; Control, fed with a basal diet; TCM 1, fed with a basal diet supplemented with 1% traditional Chinese medicine prescriptions 1; TCM 2, fed with a basal diet supplemented with 1% traditional Chinese medicine prescriptions 2.

Effects of TCM on abdominal and hepatic fat percentage

Compared to the control group, the abdominal fat percentage (Figure 5) in TCM 1 and TCM 2 groups on the 60th day decreased by 9.69% ($p<0.05$) and 3.31% ($p>0.05$) respectively, but no differences were noticed on the 30th day. Hepatic fat percentage (Figure 6) in TCM 1 treatments on the 30th and 60th days decreased by 14.98% ($p<0.05$) and 28.68% ($p<0.01$), respectively, which in dietary supplementation TCM 2 on the 30th and 60th days decreased by 8.60% ($p>0.05$) and 26.09% ($p<0.01$), respectively.

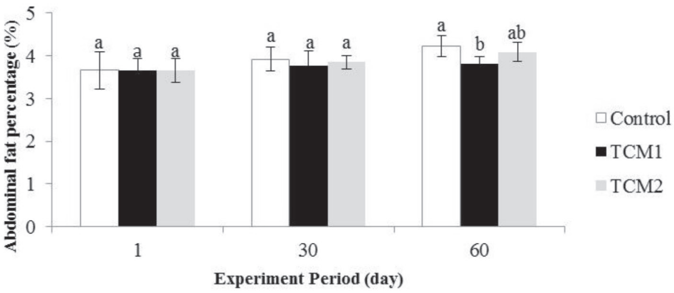

Figure 5 – Abdominal fat percentage (%). ^{a,b,c} $p<0.05$. $^{A, B, C}$ $p<0.01$. TCM, traditional Chinese medicine prescriptions; Control, fed with a basal diet; TCM 1, fed with a basal diet supplemented with 1% traditional Chinese medicine prescriptions 1; TCM 2, fed with a basal diet supplemented with 1% traditional Chinese medicine prescriptions 2.

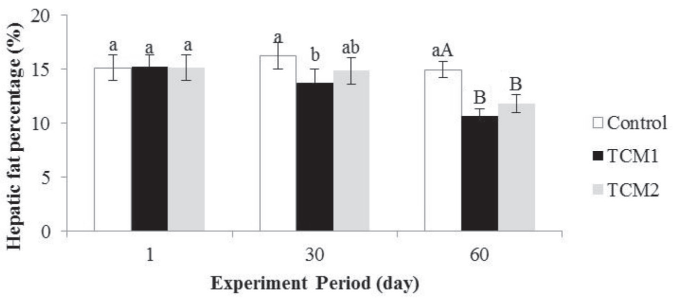

Figure 6 – Hepatic fat percentage. ^{a,b,c} $p<0.05$. $^{A, B, C}$ $p<0.01$. TCM, traditional Chinese medicine prescriptions; Control, fed with a basal diet; TCM 1, fed with a basal diet supplemented with 1% traditional Chinese medicine prescriptions 1; TCM 2, fed with a basal diet supplemented with 1% traditional Chinese medicine prescriptions 2.

Effects of TCM on expression levels of ApoA I and $ApoB_{100}$ mRNA in the Liver

Compared to the control group, dietary supplementation with TCM 1 increased the expression level of apoA I mRNA (Figure 7) on the 30th and 60th days by 38.79% ($p<0.01$) and 41.75% ($p<0.01$) respectively, while dietary supplementation with TCM 2 increased by 19.83 % ($p<0.05$) and 14.56% ($p<0.05$) respectively. The expression level of $apoB_{100}$ mRNA (Figure 8) in TCM 2 group increased by 27.3% ($p<0.01$) and 14.86% ($p<0.05$) respectively on the 30th and 60th days, dietary supplementation with TCM 1 increased by 12.84% ($p<0.05$) on the 60th day, but no differences were noticed on the 30th day.

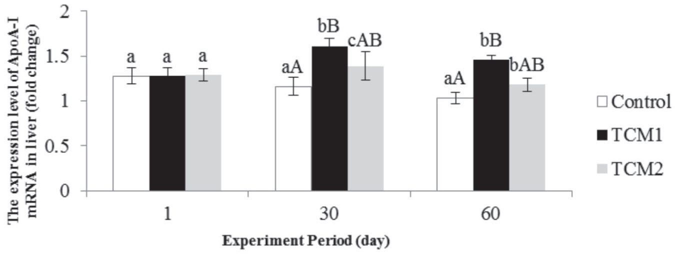

Figure 7 – The expression level of apoA I mRNA in liver. ^{a,b,c} $p<0.05$. $^{A, B, C}$ $p<0.01$. TCM, traditional Chinese medicine prescriptions; Control, fed with a basal diet; TCM 1, fed with a basal diet supplemented with 1% traditional Chinese medicine prescriptions 1; TCM 2, fed with a basal diet supplemented with 1% traditional Chinese medicine prescriptions 2.

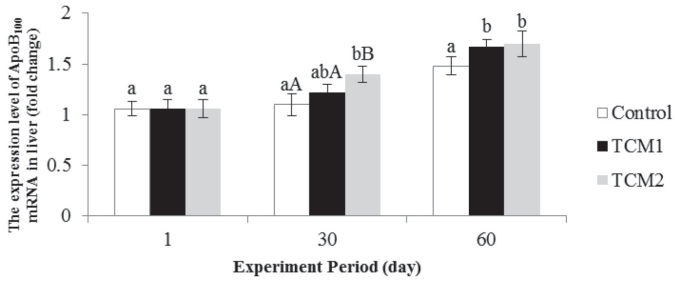

Figure 8 – The expression level of $apoB_{100}$ mRNA in liver. ^{a,b,c} $p<0.05$. $^{A, B, C}$ $p<0.01$. TCM, traditional Chinese medicine prescriptions; Control, fed with a basal diet; TCM 1, fed with a basal diet supplemented with 1% traditional Chinese medicine prescriptions 1; TCM 2, fed with a basal diet supplemented with 1% traditional Chinese medicine prescriptions 2.

DISCUSSION

In the present study, the changes of laying performance, serum lipid, hepatic and abdominal fat percentage, and mRNA expression level of apoA I, apoB genes were investigated. The improvement in egg production due to TCM supplementation was observed. Chinese herbal medicine which contained a certain amount of nutrients and some unknown growth-promoting active substances could enhance appetite, and promote body metabolism (Cai *et al.*, 2004). But average daily laying rate in TCM 1 treatment increased significantly when compared to the dietary supplemented with TCM 2. A possible reason is that Codonopsis pilosula (Lin *et al.*, 2014) is generally well-known for tonifying qi, and Angelica sinensis is an important drug to nourish blood (Yang *et al.*, 2009). Besides, the Poria presented strong kidney protection, which stated that TCM 1 could significantly strengthen the ovarian function of layers (He *et al.*, 2014; Zhao *et al.*, 2013).

In this experiment, the results showed that both TCM 1 and TCM 2 declined the levels of serum lipid and blood lipid significantly, and it might be due to the TCMs which contained the ingredients that can regulate lipid metabolism. The earlier studies showed that the serum lipid level decreased significantly in high-fat rabbit diet by providing rabbit with Crataegus pinnatifida which is rich in total flavonoids from Crataegus pinnatifida, meanwhile, it has been confirmed that Astragalus membranaceus and Codonopsis pilosula could protect the liver and the heart, which also benefits for lipid metabolism (Jiang *et al.*, 2013; Li *et al.*, 2012; Wu *et al.*, 2014). Moreover, some reports showed that Lotus leaf leads to a decrease in the levels of serum lipid (Cho *et al.*, 2007; Kim *et al.*, 2013). Furthermore, many studies uncovered that TCM could affect total lipid metabolism by reducing serum lipid level (Guo *et al.*, 2015; Kwon *et al.*, 2005; Tu *et al.*, 2003). The present study showed a significant decrease in hepatic fat percentage and a decrease trending in abdominal fat percentage after the dietary supplemented with TCM. It indicated that TCM could promote the oxidation and decomposition of fatty acid to prevent the deposit of fat in visceral.

ApoA I is the major protein of HDL, and the level of serum HDL-C is a main indicator to assess the ability of HDL facilitating the transport of lipid from peripheral tissues to the liver for disposal (Barter, 2011). The present data showed that the expression level of apoA I mRNA increased significantly in two TCM treatment groups. Compared to the control group, the data was consistent with the change trend of HDL-C in this experiment, which was in accordance with the previous study (Chor *et al.*, 2009). These results clearly stated that both of TCM 1 and TCM 2 played important roles in scavenging serum lipid. The serum TG and TC levels had a significant decrease. $ApoB_{100}$ is a constituent of LDL particles, and the increased secretion of $apoB_{100}$ contributes to the removal of lipid from the liver (Srivastava *et al.*, 2000). The results presented that the dietary supplemented with TCM 1 and TCM 2 increased the expression level of $apoB_{100}$ mRNA, which reduced the probability of the fat deposit in the liver and hepatic steatosis. These changes may be associated with the effects of *Astragalus membranaceus* that were against liver injury (Sun *et al.*, 2008). The decreasing of cholesterol content in eggs was observed in both TCM treatments, which follows with the change of serum TC levels. In layers, cholesterol was incorporated into VLDL particles after biosynthesis in the liver. As the hens were sexually mature, massive amounts of VLDL particles, including TC, were produced and secreted into serum by liver under the stimulation of estrogen. And then the VLDL particles escape through lipolysis, and the TC was transferred from the serum to the eggs (Zhou *et al.*, 2014). It suggested that reduced serum TC level apparently alleviated the cholesterol deposition in eggs. This could, in turn, explain the decrease of serum TC level and cholesterol content.

CONCLUSION

In conclusion, dietary supplemented with TCM exerts positive influences on lipid metabolism. These effects could be helpful for the control of lipid metabolism diseases in laying hens and human beings also could benefit from the better productions.

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