Effects of Chitosan on Serum Lipids and Adipocytokines in Layer Breeder Chickens: Postprint

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Abstract

This experiment aimed to investigate the effects of dietary supplementation with different levels of chitosan on lipid substances and adipocytokine contents in the serum of laying breeder hens. A total of 450 healthy 26-week-old Hy-Line Brown laying breeder hens were selected and randomly divided into 5 groups with 6 replicates per group and 15 hens per replicate. The control group was fed a basal diet without chitosan supplementation, while the four experimental groups were fed experimental diets supplemented with 250, 500, 1,000, and 2,000 mg/kg chitosan in the basal diet, respectively. The experimental period lasted 56 days. The results showed that: compared with the control group, dietary supplementation with 250, 500, 1,000, and 2,000 mg/kg chitosan could reduce the contents of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), free fatty acids (FFA) in serum and liver FFA content in laying breeder hens to varying degrees on days 28 and 56 of the experiment. On day 28 of the experiment, compared with the control group, dietary supplementation with 250, 500, and 1,000 mg/kg chitosan significantly reduced triglyceride (TG) content in serum (P 0.05), supplementation with 500 mg/kg chitosan significantly reduced very low-density lipoprotein (VLDL) content in serum (P 0.05), supplementation with 250, 500, 1,000, and 2,000 mg/kg chitosan significantly reduced leptin (LEP) content in serum (P 0.05), and supplementation with 250 mg/kg chitosan significantly increased high-density lipoprotein cholesterol (HDL-C) content in serum (P 0.05). On days 28 and 56 of the experiment, serum TC content in laying breeder hens showed a significant linear decreasing relationship with chitosan supplementation level ($P<0.01$); on day 28 of the experiment, serum TG ($P<0.01$), HDL-C ($P<0.01$), FFA ($P=0.04$), and VLDL contents in laying breeder hens showed a significant quadratic relationship with chitosan supplementation level, and regression analysis indicated that chitosan supplementation levels of 652.56–967.18 mg/kg had a better regulatory effect on the above indicators. It can be concluded that dietary chitosan supplementation can improve

the health status of lipid metabolism in laying breeder hens, and its effects on serum lipid substance contents and liver FFA content in laying breeder hens are related to its supplementation level.

Full Text

Effects of Chitosan on Serum Lipid and Adipocytokine Contents of Laying Breeders

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Abstract: This experiment was conducted to investigate the effects of dietary chitosan supplementation at different levels on serum lipid and adipocytokine contents in laying breeders. Four hundred and fifty healthy 26-week-old Hy-Line Brown laying breeders were randomly assigned to 5 groups with 6 replicates per group and 15 chickens per replicate. The control group was fed a basal diet without chitosan, while the four experimental groups were fed the basal diet supplemented with 250, 500, 1,000, or 2,000 mg/kg chitosan. The trial lasted for 56 days. The results showed that compared with the control group, dietary supplementation with 250, 500, 1,000, and 2,000 mg/kg chitosan could reduce the contents of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and free fatty acids (FFA) in serum and the content of FFA in liver of laying breeders to varying degrees on days 28 and 56 of the experiment. On day 28, dietary supplementation with 250, 500, and 1,000 mg/kg chitosan significantly reduced serum triglyceride (TG) content (P 0.05), supplementation with 500 mg/kg chitosan significantly reduced serum very low-density lipoprotein (VLDL) content (P 0.05), supplementation with 250, 500, 1,000, and 2,000 mg/kg chitosan significantly reduced serum leptin (LEP) content (P 0.05), and supplementation with 250 mg/kg chitosan significantly increased serum high-density lipoprotein cholesterol (HDL-C) content (P 0.05). On days 28 and 56, serum TC content showed a significant linear decreasing relationship with chitosan supplementation level ($P<0.01$). On day 28, serum TG ($P<0.01$), HDL-C ($P<0.01$), FFA ($P=0.04$), and VLDL contents ($P<0.01$) showed significant quadratic relationships with chitosan supplementation level. Regression analysis indicated that chitosan supplementation levels of 652.56–967.18 mg/kg provided better regulation of these indices. It is concluded that dietary chitosan supplementation can improve the health status of lipid metabolism in laying breeders, and its effects on serum lipid contents and liver FFA content are related to its supplementation level.

Keywords: chitosan; laying breeders; lipid metabolism; free fatty acids; very low-density lipoprotein; adipocytokine

Introduction

The egg is the sole source of nutrients for chicken embryo development, and its energy content is the first limiting factor for embryonic growth. While egg protein is primarily utilized for tissue growth, lipids in the yolk serve as the main energy source for embryonic development. Laying breeders exhibit active lipid metabolism to meet the demands of yolk lipid deposition. Therefore, effective regulation of lipid metabolism in laying breeders is crucial for improving their health status and production performance.

Serum lipid and adipocytokine contents are important indicators reflecting animal lipid metabolism. Research has shown that chitosan and its derivatives, due to their biologically active functional groups such as amino and hydroxyl groups, can regulate lipid metabolism when appropriately supplemented in animal diets. However, relevant reports on the effects of chitosan on lipid metabolism in poultry are limited. Zhou et al. found that dietary supplementation with 0.14% or 0.28% chitosan could reduce abdominal fat percentage, increase blood HDL-C content, improve breast meat quality, decrease total saturated fatty acid content in breast muscle, and increase monounsaturated fatty acid content in broilers. Kobayashi et al. reported that supplementation with 0.5% chitosan in high-fat diets had no significant effect on growth performance but could reduce abdominal fat percentage and small intestinal lipase activity in chickens. Nevertheless, some studies have reported different results. Keser et al. found that supplementation with 0.025% chitosan in broiler diets only reduced blood LDL-C content without significantly affecting growth performance or blood TC, HDL-C, or TG contents. Given the inconsistent conclusions among researchers and the variation in supplementation levels, particularly the scarcity of research on laying breeders, this experiment was designed to investigate the effects of different dietary chitosan levels on serum lipid and adipocytokine contents in laying breeders, thereby exploring its influence on lipid metabolism and providing a theoretical basis for further research on the mechanisms and improvement of lipid metabolism in laying breeders.

1.1 Experimental Animals, Diet Composition, and Management

Four hundred and fifty healthy 26-week-old Hy-Line Brown laying breeders were selected and randomly divided into 5 groups according to the principle of similar body weight and laying rate, with 6 replicates per group and 15 chickens per replicate. The control group was fed a basal diet without chitosan, while the four experimental groups were fed the basal diet supplemented with 250, 500, 1,000, or 2,000 mg/kg chitosan. The chitosan used in the experiment was provided by Jinan Haidebei Marine Bioengineering Co., Ltd. (Shandong) with a deacetylation degree of 84.15% and viscosity of 45 cps. The experimental period lasted for 56 days. The basal diet was formulated according to the NRC (1994)

nutrient requirements for laying breeders, and its composition and nutrient levels are shown in Table 1 .

Laying breeders were housed in a windowed enclosed chicken house with three-tier cage systems. During the experimental period, they had free access to feed and water with 16 hours of daily lighting. Environmental conditions and management were consistent across all groups, with regular disinfection and conventional immunization programs.

1.2 Sample Collection and Index Determination

1.2.1 Sample Collection and Processing On days 28 and 56 of the experiment, one chicken was randomly selected from each replicate, and 4 mL of blood was collected from the wing vein per chicken. The blood was centrifuged at 1,000×g for 10 minutes, and the supernatant was collected and stored at -20 °C for later use. After blood collection, the chickens were slaughtered, and the liver was excised, rinsed with precooled physiological saline to remove surface blood stains, and stored at -20 °C.

1.2.2 Determination Indices and Methods Serum TG and TC contents were determined using the single-reagent glycerol phosphate oxidase-peroxidase (GPO-PAP) method. Serum HDL-C and LDL-C contents were measured using the double-reagent direct method. Serum and liver free fatty acid (FFA) contents were determined by colorimetric assay. Serum leptin (LEP) and adiponectin (ADP) contents were measured by enzyme-linked immunosorbent assay (ELISA). Serum very low-density lipoprotein (VLDL) content was determined by radioimmunoassay. All these indices were measured using commercial kits purchased from Nanjing Jiancheng Bioengineering Institute, with specific procedures performed according to the kit instructions.

Table 1 Composition and nutrient levels of the basal diet (air-dry basis)

Ingredients	Content	Nutrient levels[2])	Content
Corn		Metabolizable energy (ME)/(MJ/kg)[2])	
Soybean meal		Crude protein (CP)	
Shell meal		Available phosphorus (AP)	
DL-Met		Methionine (Met)	
Bone meal		Lysine (Lys)	
Choline chloride		Tryptophan (Try)	
NaCl			
Premix[1])			
Total			

[1]) The premix provided the following per kg of the diet: Mn 50 mg, Fe 25 mg, Cu 2.5 mg, Zn 50 mg, I 1.0 mg, Se 0.15 mg, VA 7,715 IU, VD 2,755 IU, VE

8.8 IU, VK 2.2 mg, VB 10.55 mg, VB 8.0 mg, VB 24.41 mg, VB 120.01 mg, nicotinic acid 19.8 mg, folic acid 0.28 mg, biotin 2 mg, calcium pantothenate 50 mg.
[2]) ME was a calculated value, while the others were measured values.

1.3 Statistical Analysis

Experimental data were analyzed using SAS 9.1.3 software for one-way ANOVA and linear and quadratic regression analysis. P 0.05 indicated significant differences between groups or regression relationships, while 0.05<P<0.10 indicated a trend toward significance.

2.1 Effects of Chitosan on Serum Lipid Contents and Liver FFA Content of Laying Breeders

As shown in Table 2 , on day 28, serum TG content in the 250, 500, and 1,000 mg/kg chitosan groups was significantly lower than that in the control group (P 0.05), with the 500 mg/kg group showing the lowest value, while the 2,000 mg/kg group showed no significant difference from the control group (P>0.05). With increasing chitosan supplementation levels, serum TG content exhibited a significant quadratic change (R^2=0.8399, P<0.01). On day 56, serum TG content in the 250 mg/kg chitosan group was significantly lower than that in the control group (P 0.05), and the 500 mg/kg group also showed a decreasing trend, though not significantly different from the control group (P>0.05).

Except for the 250 mg/kg chitosan group on day 56, serum TC content in all chitosan groups on both days 28 and 56 was significantly lower than that in the control group (P 0.05). Moreover, with increasing chitosan supplementation levels, serum TC content showed a significant linear decrease on both days 28 (R^2=0.6490, P<0.01) and 56 (R^2=0.5684, P<0.01). On day 28, serum HDL-C content in the 250 mg/kg chitosan group was significantly higher than that in the control group (P 0.05), while the 500, 1,000, and 2,000 mg/kg groups showed no significant difference from the control group (P>0.05). Serum HDL-C content exhibited a significant quadratic change with increasing chitosan supplementation levels (R^2=0.8555, P<0.01). On day 56, no significant differences in serum HDL-C content were observed between any chitosan group and the control group (P>0.05).

On day 28, serum LDL-C content in the 250 mg/kg chitosan group was significantly lower than that in both the control and 2,000 mg/kg groups (P 0.05). On day 56, serum LDL-C content in all chitosan groups was significantly lower than that in the control group (P 0.05). On day 28, serum VLDL content in the 500 mg/kg chitosan group was significantly lower than that in all other groups (P 0.05), and the 250 mg/kg group was significantly lower than the 1,000 and 2,000 mg/kg groups (P 0.05). With increasing chitosan supplementation levels, serum VLDL content showed a significant quadratic change (R^2=0.4077,

P<0.01). On day 56, serum VLDL content in all chitosan groups was lower than that in the control group, though the differences were not significant (P>0.05).

On day 28, serum FFA content in the 250, 500, and 1,000 mg/kg chitosan groups was significantly lower than that in the control group (P 0.05), showing a significant quadratic relationship with chitosan supplementation level (R^2=0.3592, P=0.04). On day 56, serum FFA content in the 250 and 500 mg/kg chitosan groups was significantly lower than that in the control group and other chitosan groups (P 0.05). On day 28, liver FFA content in the 250 and 500 mg/kg chitosan groups was significantly lower than that in the control group (P 0.05), with the 250 mg/kg group showing the lowest value, while the 1,000 and 2,000 mg/kg groups showed no significant difference from the control group (P>0.05). On day 56, liver FFA content in the 250 mg/kg chitosan group was significantly lower than that in the control group (P 0.05), while other chitosan groups showed no significant difference from the control group (P>0.05).

Table 2 Effects of chitosan supplementation on serum lipid contents and liver free fatty acid content of laying breeders

Note: In the same row, values without the same small letter superscripts mean significant difference (P 0.05), while values with the same letter superscripts mean no significant difference (P>0.05). The same as below.

2.2 Effects of Dietary Chitosan Supplementation on Serum Adipocytokine Contents of Laying Breeders

As shown in Table 3 , on day 28, dietary supplementation with 250, 500, 1,000, and 2,000 mg/kg chitosan all significantly reduced serum LEP content (P 0.05). On day 56, supplementation with 250, 500, 1,000, and 2,000 mg/kg chitosan had no significant effect on serum ADP content (P>0.05).

Table 3 Effects of chitosan supplementation on serum adipocytokine contents of laying breeders

Discussion

Serum contents of TG, TC, HDL-C, and LDL-C are important indicators of lipid metabolic health in animals. Elevated TG content increases the risk of blood coagulation, promotes atherosclerosis formation and development, and leads to complications such as fatty liver disease and obesity. High TC content tends to accumulate in arterial walls, causing atherosclerosis. The results of this study indicate that dietary chitosan supplementation can reduce serum TG, TC, and LDL-C contents and increase HDL-C content in laying breeders, suggesting that chitosan supplementation is beneficial for improving serum lipid metabolic health.

The earliest proposed mechanism for chitosan' s lipid-regulating effect is the lipid-binding mechanism. The positive charges on chitosan' s amino groups

promote its binding to anionic substances such as fatty acids and bile acids, interrupting intestinal lipid absorption and promoting bile acid excretion. To compensate for fecal losses, TC in the liver is acceleratedly converted to bile acid, thereby exhibiting lipid- and cholesterol-lowering effects. HDL-C has the function of reverse-transporting cholesterol to the liver, leading to increased plasma HDL-C content. LDL-C is the main cholesterol carrier in blood, transporting cholesterol to peripheral tissues and regulating de novo cholesterol synthesis in these locations, thereby promoting fat deposition. Additionally, chitosan is considered a competitive inhibitor of pancreatic lipase, reducing lipid and cholesterol absorption. Therefore, chitosan can reduce serum TG and TC contents in laying breeders, though the exact mechanisms require further investigation.

The results also demonstrate that the cholesterol-lowering effects of chitosan are dose-dependent. Dietary supplementation with 250, 500, and 1,000 mg/kg chitosan showed greater TG-reducing effects. On day 28, regression analysis indicated that a chitosan supplementation level of 967.18 mg/kg resulted in the lowest serum TG content. With increasing chitosan supplementation levels, serum TC content was linearly decreased. Furthermore, 250 and 500 mg/kg chitosan showed superior ability to regulate serum HDL-C and LDL-C contents compared with 1,000 and 2,000 mg/kg chitosan, suggesting that low-dose chitosan has better regulatory effects on lipid metabolism than high doses. On day 28, regression analysis showed that a chitosan supplementation level of 734.61 mg/kg produced the highest serum HDL-C content.

The liver is the primary site of lipid synthesis in poultry. Imbalance between TG synthesis and decomposition in the liver leads to excessive TG deposition in hepatocytes, inducing fatty liver disease and decreasing laying rate. Compared with de novo synthesis and dietary fatty acids, FFA can be directly incorporated into VLDL-TG. Koutsari et al. demonstrated that FFA is the main fatty acid source for VLDL-TG. Other studies have also confirmed that FFA is the primary fatty acid source for liver TG synthesis. Therefore, increased availability of fatty acids in the liver is the key point for excess liver TG. In this study, dietary chitosan supplementation reduced liver FFA content in laying breeders, decreasing the total amount of available TG synthesis and appropriately limiting excessive TG synthesis in the liver due to laying demands, thereby preventing massive TG deposition in hepatocytes. Additionally, the liver' s capacity to absorb FFA is proportional to its blood concentration. This study showed that dietary chitosan supplementation reduced serum FFA content in laying breeders, which would affect liver FFA uptake and consequently reduce liver FFA content.

Moreover, esterification of FFA to form TG can prevent hepatocellular injury or dysfunction caused by fatty acid deposition. The chronic adverse effects of exposing non-adipose cells and tissues to high fatty acid levels are termed "lipotoxicity," primarily caused by the inability to esterify fatty acids. Therefore, dietary chitosan supplementation reduces serum and liver FFA contents in laying breeders, which not only limits excessive TG deposition in the liver but also alleviates the cytotoxicity of FFA, prevents liver injury, and improves

lipid metabolic health. On day 28, dietary supplementation with 250, 500, and 1,000 mg/kg chitosan significantly reduced serum FFA content, with regression analysis indicating that a chitosan level of 856.67 mg/kg produced the lowest serum FFA content. Additionally, 250 and 500 mg/kg chitosan showed better FFA-reducing effects in serum and liver on day 56 than 2,000 mg/kg chitosan. These results demonstrate that the FFA-lowering effects of chitosan are dose-dependent, with low-dose groups showing better regulatory effects and high-dose groups showing diminished effects.

Lipid transport in the body is primarily accomplished through lipoprotein particles such as VLDL and chylomicrons. Elevated VLDL content can meet the vigorous lipid demands for egg production after hens reach sexual maturity, but high VLDL content can also induce fatty liver disease and affect laying performance. Blood lipoprotein content results from the balance between "appearance rate" and "clearance rate" of lipids in blood. An Guangquan found that as serum VLDL content increased, laying rate first increased then decreased; when serum VLDL content was less than 400 mg/mL, it was significantly positively correlated with laying rate, but when greater than 400 mg/mL, it was significantly negatively correlated. Chen Yuanyuan reported similar conclusions. Our previous research showed that the laying rates of the 250 and 500 mg/kg chitosan groups were 94.14% and 95.19%, respectively, while those of the control, 1,000, and 2,000 mg/kg groups were 90.52%, 93.06%, and 93.30%, respectively, which was opposite to the pattern of serum VLDL content changes observed in this study. This suggests that different chitosan doses may affect laying performance by regulating serum VLDL content. However, on day 56, dietary chitosan supplementation had no significant effect on serum VLDL content, possibly because serum VLDL content needs to remain high during peak laying periods to meet lipid demands for egg production. The exact mechanism requires further investigation. Additionally, chitosan regulation of serum VLDL content showed dose-dependent effects, with regression analysis on day 28 indicating that a chitosan supplementation level of 652.56 mg/kg produced the lowest serum VLDL content.

These results demonstrate that chitosan regulation of serum TG, VLDL, HDL-C, and FFA contents in laying breeders exhibits dose-dependent effects. Based on regression analysis of these indices, the optimal chitosan supplementation levels were 967.18, 652.56, 734.61, and 856.67 mg/kg, respectively. This indicates that chitosan supplementation at 652.56–967.18 mg/kg provides better regulation of serum TG, HDL-C, VLDL, and FFA contents, further confirming that high-dose chitosan supplementation diminishes regulatory effects on lipid metabolism. However, the optimal dosage for lipid metabolism regulation requires further investigation with additional dose groups between 500–1,000 mg/kg.

Leptin (LEP) is a polypeptide hormone encoded by the obesity gene and synthesized and secreted by white adipocytes. Its most fundamental role is regulating fat metabolism and reducing body fat deposition. LEP-resistant mice exhibit hypercholesterolemia, hypertriglyceridemia, hepatic steatosis, and impaired fat

tolerance. In obese mice and humans, serum LEP content is proportional to body fat mass and positively correlated with TC, TG, VLDL, and LDL-C contents, but negatively correlated with HDL-C content. LEP-resistant mice lacking hepatic LEP signaling show abnormal plasma lipoprotein remodeling and increased VLDL-TG content. Given the liver' s key role in lipid metabolism, researchers believe that increased dyslipidemia risk is caused by hepatic LEP resistance. In this study, dietary chitosan supplementation at different levels significantly reduced serum LEP content in laying breeders, indicating that chitosan' s regulatory effects on serum TC, TG, VLDL, LDL-C, and HDL-C contents are related to LEP.

Furthermore, high LEP content stimulates TG hydrolysis and fatty acid release and oxidation while reducing total fatty acid uptake. LEP increases lipolysis by enhancing triglyceride lipase and hormone-sensitive lipase activities, releasing FFA. Jaubert et al. found that LEP stimulates nitric oxide production, thereby inhibiting glycerol synthesis and reducing fatty acid re-esterification opportunities. William et al. incubated adipocytes under LEP conditions and used radiolabeling techniques to measure fatty acid influx (fatty acid synthesis to TG) and efflux (intracellular fatty acid oxidation and FFA release), finding that TG hydrolysis rate increased by 123% (measured by FFA release) and net fatty acid efflux increased by 30%. In this study, serum and liver FFA contents in chitosan groups were lower than those in the control group to varying degrees, which may be related to LEP. Current research shows that LEP reduces lipogenesis by inhibiting sterol regulatory element-binding protein 1 and peroxisome proliferator-activated receptor and their regulated lipogenic enzymes and mRNA expression, while promoting lipolysis and fatty acid oxidation by upregulating lipolytic and fatty acid oxidative enzymes. Therefore, further research is needed on the important role and mechanisms of LEP in chitosan-regulated lipid metabolism in laying breeders.

Conclusion

1. Dietary chitosan supplementation can reduce serum TG, TC, LDL-C, and FFA contents and liver FFA content while increasing serum HDL-C content in laying breeders, thereby improving their lipid metabolic health status.
2. Dietary chitosan supplementation also demonstrates certain leptin-lowering effects in laying breeders.
3. Dietary chitosan supplementation levels of 652.56–967.18 mg/kg provide better regulation of serum TG, LDL-C, VLDL, and FFA contents in laying breeders.

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Note: Figure translations are in progress. See original paper for figures.