

Quantitative detection of mrna by real time pcr biology essay

[Science](#), [Biology](#)



Abstract

The aims of the present study were to evaluate the effects of dietary phytosterols (PS) on growth performance and lipid metabolism in broilers. Four hundred, one day old broiler chickens were randomly divided into 4 groups fed a corn soya based broiler diets supplemented without PS (control) or with PS 30, 40, and 50 mg/kg of diet for 42 days of age. There was no effect of PS supplementation on growth performance and serum triglycerides. The serum free fatty acid was significantly increased in both female and male chickens supplemented with PS 40 and 50mg/kg of diet, respectively. The serum leptin, insulin and hormone sensitive lipase activity were significantly increased ($P < 0.05$) in all PS supplemented diets as compared to control group. However, 40 and 50 mg/kg diet of PS had more influenced on serum leptin and insulin in female and male broilers, respectively. The PS supplemented diets significantly decreased ($P < 0.05$) mRNA expression of hepatic fatty acid synthase enzyme in both male and female chickens. The mRNA expression of sterol regulatory element-binding transcription factor 1c was significantly decreased in the diets supplemented with PS 40 and 50mg/kg in both female and male chickens. Therefore, the results suggested that the PS supplemented diets regulated lipid metabolism in broilers. The poultry diets supplemented with PS 40mg/kg of diet were down-regulated fat deposition in females, while, PS 50 mg/kg of diets promoted lipolysis in male chickens. Economic prosperity amongst masses has increasingly improved their living standards (Nordhaus, 2002).

Consumers are growingly yearning for health-promoting effects of functional foods enriched with natural ingredients, given situation have led to the

mechanisms for commercializing meat and its by-products with improved standards simultaneously (Grashorn, 2007). However, animal breeding with modern intensive production system leads to make high energy levels for broilers, which is easy to cause an excess of fat deposition. Phytosterols (PS) are plant-derived active substances found in fruits, vegetables, nuts, seeds, cereals, legumes, vegetable oils and other plants (Ling and Brough, 2007). They are structurally similar to cholesterol; however, the intestinal absorption of PS has been shown to be limited in both human and animals. In intestine, they compete with cholesterol for absorption into micelles and thereby decrease the solubility of cholesterol resulting in inhibition of both dietary and endogenously produced cholesterol. It is well known that they are also responsible for lowering blood cholesterol levels that is one of the major risks for cardiac problems (Chen et al., 2010; Klingberg et al., 2008). Previous studies suggested that PS enriched foods have a protective effect against cardiovascular diseases, inflammation, cancer and improve antioxidant capacity in human (Rubis et al., 2010; Jong et al., 2008; Rudkowska, 2010). The U. S Food and Drug administration was the first to approve a health claim for PS in 2000 (Food and Drug Administration, 2000), and Ministry of Agriculture of China has also approved PS as a new type of additive in 2008. In rats, PS supplemented diets leads to decrease levels of hepatic saturated fatty acid, adipose and serum phospholipid and increase 18: 2 n-6 in adipose tissues (Katamoto et al., 1991). In Another study, it was found that PS supplemented diets increased n-3 and n-6 long-chain PUFA in rat liver, testes and prostate phospholipids (Awad et al., 1997). However, scientific information available on lipid metabolism in chickens

supplemented with PS diets on both male and female sexes is limited, and often not conclusive. The objective of this study to investigate the optimize PS levels in poultry diets on growth performance, serum lipid, lipid metabolic enzymes and hepatic mRNA expressions of fatty acid synthase(FAS) and sterol regulatory element binding protein-1c (SREBP-1c) in female and male broilers chickens.

Phytosterols

PS was purchased from Jiangsu Spring Fruit Biological Products Co. Ltd, PR. China, composed of PS $\geq 91\%$, included β -sitosterol 40%-45%, stigmasterol $\geq 17\%$ and brassinostsroids $\geq 20\%$.

Birds, housing and management

Four hundred one-day-old Arbor Acres broilers were obtained from a commercial hatchery. The chickens were randomly allotted into 4 treatments consisting of 5 replicates with 20 birds (10 females and 10 males) per replicate. The chickens were fed on corn soy based diets (NRC, 1994). These diets (Table 1) supplemented without PS (control) or with different levels of PS 30, 40, 50 mg/kg of diet. The birds were vaccinated against Newcastle and infectious bursal diseases at 7, 14, and 21 d of age. The birds were kept in wire cages in a three level battery (90 cm x 70 cm x 45 cm) and temperature of the room was maintained at 34-36°C during 1 to 14 d of age and then temperature was gradually reduced to 26°C, after which it was maintained at room temperature and kept constant throughout the experiment. Chickens received a continuous lighting pattern 24 h light each day and feed and water were provided ad libitum.

Growth performance

Body weights and feed intake of chickens were recorded for each replicate at 1, 21, and 42 d of age to calculate feed conversion ratios.

Sample collection

At 42 d of age, two chickens (1 male and 1 female) per replicate were randomly selected and weighed after feed deprivation for 12 h. Blood samples were collected and serum was separated by centrifugation at 350 x g for 15 min at 4°C. The serum was collected and stored at -20°C for further analysis. After collection of blood samples, all chickens were humanely killed by exsanguination. After decapitation, livers tissue samples were excised and stored at -80°C until further analysis for mRNA expressions of FAS and SREBP-1c enzymes.

Serum analysis

Serum free fatty acids (FFA) and triglycerides (TG) were determined with commercial kits (Nanjing Jiancheng Biochemical Reagent Co., Nanjing People's Republic of China) according to the manufacturer's instructions. Plasma insulin was measured by RIA with guinea pig anti-porcine insulin serum (3V Bio-engineering group Co., Weifang, People's Republic of China). The sensitivity of the assay was 1 mIU/ml, and all samples were included in the same assay to avoid interassay variability. The intra-assay coefficient of variation (CV) was 6.9% (Song et al., 2011). Plasma leptin was measured with a commercial multi-species RIA kit purchased from Beijing North Institute of Biotechnology (Beijing, People's Republic of China). The detection limit for leptin was 0.45 ng/mL. The intra- and inter-assay coefficients of

variation were 5% and 10%, respectively. The commercial RIA kit was previously validated for measuring chicken samples (Li et al., 2007).

Enzyme activities analysis

Hormone sensitive lipase (HSL) and fatty acid synthase (FAS) enzymes activities were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions, the kits were purchased from Shanghai Blue gene Biotech Co., Ltd. People's Republic of China.

Quantitative detection of mRNA by Real-Time PCR

Total RNA was extracted from liver tissue by using TRIZOL reagent according to manufacturer's protocol, and quantified by measurement of optical density at 260 nm. Ratios of absorption (260/280 nm) of all samples were between 1.8 and 2.0. Aliquots of RNA samples were subjected to electrophoresis in a 1% ethidium bromide stained 1.4% agarose formaldehyde gel to verify their integrity. Reverse transcription was performed using 2 µg of total RNA: 5.0 µg 5×RTbuffer, 1.0 µg 106RT Random Primer (Promega, Belgium), 2 µl 256dNTP (Promega, Belgium), 0.5 µl Multiscribe Reverse Transcriptase (Promega, Belgium), 0.2 µl RNase inhibitor (Promega, Belgium), and the addition of nuclease free water to make final volume of 25 µl. Reaction system was run at 37°C for 60 min and 95°C for 5 min. Quantitative RT-PCR was performed in a 25 µl reaction buffer that included 12.5 µl SYBR GREEN, 0.25 µl of forward primer, 0.25 µl of reverse primer, 2 µl of cDNA, and 10 µl ddH₂O were incubated in a Strategene MX3000PTM Detection System (Applied Biosystems). The reaction mixture was subject to program to conduct one cycle (95°C for 30 s) and 40 cycles

(9°C for 5 s and 60°C for 31 s). The primer sequences are listed in Table 2. Each sample was assayed in duplicate and normalized to β -actin expression in liver tissues. The results (fold changes) were expressed as $2^{-\Delta\Delta C(t)}$ with $\Delta\Delta C(t) = [C(t)_{ij} - C(t)_{\beta\text{-actin}j}] - [C(t)_{i1} - C(t)_{\beta\text{-actin}1}]$, where $C(t)_{ij}$ and $C(t)_{\beta\text{-actin}j}$ are the C_t for gene i and for β -actin $_j$ in a pool or a sample (named j) and where $C(t)_{i1}$ and $C(t)_{\beta\text{-actin}1}$ are the C_t in pool 1 or sample 1, expressed as the standard.

Statistical Analysis

The data of growth performance was analyzed by one-way ANOVA using the SPSS program (version 16.0). Duncan's multiple range test ($P \leq 0.05$) was applied to test for significant differences between means. Other data was analyzed by ANOVA procedures appropriated for a randomized complete block design by the GLM procedures and differences among treatment means were determined by Duncan's new multiple range test, comparing the diets and genders factors and the interaction between these two factors. The significance was defined at $P < 0.05$ and $P < 0.01$.

Effect of dietary PS on growth performance in AA broilers

The results of growth performance in AA broiler chickens that has been fed diets supplemented without PS or with PS are shown in Table 3. There was no significant difference ($P < 0.05$) on average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) in broiler chickens among all experimental groups either supplemented with different levels of PS or without PS supplementation during 42 d of growth period.

Effect of dietary PS on serum lipid of female and male AA broilers

The results of serum FFA and TG are shown in Table 4. Significant main effects of diets ($P < 0.05$) and sexes ($P < 0.01$) on serum FFA were found in broilers after PS supplementation in poultry diets. A Significant ($P < 0.05$) interaction between diets and sex was also found for serum FFA. Male chickens exhibited higher FFA concentrations than female chickens and dietary PS significantly increased ($P < 0.05$) serum FFA concentrations than control group chickens fed diets without PS. At 42 d of age, female chickens that received PS 40mg/kg of diet had higher serum FFA concentrations while male chickens received PS 50mg/kg had higher concentrations of serum FFA. In female chickens, the level of serum FFA was increased 23.43% when supplemented with PS 40 mg/kg of diet while in male chickens the increased was 10.98% at 50 mg of PS/kg of diet as compared with the control group. There was no significant ($P < 0.05$) affect found on serum TG concentrations among all experimental groups at 42 d. Non significant ($P < 0.05$) interactions between diets and sex were also found for serum TG in broiler chickens.

Effect of dietary PS on plasma leptin and insulin of female and male AA broilers

The results of dietary PS on plasma leptin and insulin concentrations are shown in Table 5. Significant ($P < 0.05$) effects of diets, sex and interaction of both diets and sex on plasma leptin concentrations were found in chickens. PS supplemented diets significantly ($P < 0.05$) increased plasma leptin concentrations in all groups both in female and male chickens ($P < 0.05$).

05). Male chickens exhibited higher plasma leptin concentrations than female chickens. Plasma leptin concentrations was significantly highest ($P < 0.05$) in diets supplemented PS with 40 and 50mg/kg of diet in female and male chickens, respectively. In female chickens, the level of serum leptin was increased 41.04% when supplemented with PS 40 mg/kg of diet while in male chickens the increased was 50.96% at 50 mg of PS/kg of diet as compared with the control group. In our present study results showed that plasma insulin concentrations were affected by PS diets but not affected by sexes, however, significant interaction between diets and sexes were found for insulin concentrations. Plasma insulin concentrations was increased by PS diets ($P < 0.05$), particularly highest when PS supplemented with 40 and 50mg/kg of diet in female and in males, respectively. The plasma insulin was increased 133.12% in female chickens when supplemented with PS 40 mg/kg of diet while in male chickens the increased was 26.85% at 50 mg of PS/kg of diet as compared with the control group.

Effect of dietary PS on enzyme activities of female and male AA broilers

The results of dietary PS supplementation on enzyme activities of serum HSL and FAS are shown in Table 6. PS supplemented diets had a significant influenced on serum FAS enzyme activity ($P < 0.05$) but not affected by sexes, however, a significant interaction between PS supplemented diets and sexes were found on serum FAS activity in broilers at 42d of age. Dietary PS in the diets caused serum FAS in the chickens to be lower than the chickens in control group without PS in the diets. Male chickens exhibited lower FAS concentrations than female chickens. Serum FAS activity was decreased in

diets supplemented with PS 30 and 40mg/kg of diet in males and female chickens, respectively. In female chickens, the level of serum FAS activity was decreased 50.93% when supplemented with PS 40 mg/kg of diet while in male chickens the decreased was 75.23% at 50 mg of PS/kg of diet as compared with the control group chickens. Significant ($P < 0.05$) effects of diets, sexes and interaction of both diets and sexes ($P < 0.05$) on serum HSL enzyme activity were found in chickens at 42 d of age. Male chickens exhibited lower HSL concentrations than female chickens, and dietary PS in the diets caused serum HSL in the chickens to be higher than in chickens in control group given no PS supplementation in the diet. The activity of HSL enzyme had significant decreased ($P < 0.05$) in chickens supplemented with diets with PS 50mg/kg while it was increased at 40mg/kg of diets. In female chickens, the level of serum HSL enzyme activity was increased 23.91% at PS 40 mg/kg of diet while in male chickens the increased was 48.42% at 50 mg of PS/kg of diet as compared with the control group.

Effect of dietary PS on the mRNA expressions of hepatic FAS and SREBP-1c genes

Significant ($P < 0.05$) effect of PS supplemented diets on mRNA expressions of hepatic FAS and SREBP-1c genes were found but it was not affected by sexes, however, significant interaction of both diets and sexes were found in chickens at 42 d. The diets supplemented with PS significantly ($P < 0.05$), decreased hepatic mRNA gene expression of FAS enzyme in chickens. The decreased was highest when supplemented with PS 40 and 50mg/kg of diets in female and male chickens, respectively. However, mRNA expression of SREBP-1c in liver was significantly ($P < 0.05$) increased with supplementation

of PS 30mg/kg of diet in female chickens, and a slight increased in male chickens fed diets with PS 30mg/kg of diet. Furthermore, chickens supplemented with PS 40mg/kg of diet significantly ($P < 0.05$) decreased hepatic mRNA expression of SREBP-1c in female chickens, while PS supplementation of 50mg/kg of diet down-regulated the expression of SREBP-1c in liver of in male chickens. In female chickens, the decrease was 45.0% and 58.02% for SREBP-1c and FAS enzymes, respectively, while, for male chickens, the decrease was 53.39% and 71.28% as compared with the control group.

Discussion

PS are plant-derived lipids with similar chemical structure to cholesterol. Humans and animals neither synthesize nor efficiently absorb PS. The PS supplemented diets decrease the serum cholesterol by inhibiting the uptake of cholesterol and by incorporation into mixed micelles in the small intestine in human and animals. In this present study, the results showed that PS had no significant influenced on growth performance (ADFI, ADG, and FCR) during the whole growth period of 42 d. Our results are concurrence with the previous studies of Elkin and Lorenz (2009) who found that there was no effect of supplementation of PS (1g per 100 g of diet) on weight gain, feed consumption, and feed efficiency in layer hens at 28 d of age. Similarly, Liu et al. (2010) also concluded that PS supplementation had no significant effect on feed intake and body weight in layers after 8 weeks of age. However, other studies in pigs and in broilers found that diets supplemented with PS improved the growth performance in animals. The difference in

results might be due to differences in animal species, chemical composition of PS and levels of PS used in the animal diets. Serum FFA mainly released from adipose tissue and most of the fat required by other tissues, including the liver, is provided by the adipose tissue via FFA. Most of the circulating FFA is bound to albumin and involved in supplying fat to various tissues as well as for oxidation in the fasting state. In this present study, we found that PS supplementation in the poultry diets significantly improved the serum FFA both in female and male broiler chickens. Our data is concurrence with the results of Miswa et al. (2012) who found that the PS isolated from Aloe vera decreased serum FFA in Zucker diabetic fatty rats. However, studies of Katamoto et al. (1991) found that the serum FFA was unchanged in rats. The results suggested that PS supplementation facilitates the release of FFA in the bloodstream and accelerating the fat mobilization more efficiently in females (40 mg/kg of diet) as compared to males (50 mg/kg of diet). There was no significant difference in serum TG among all experimental groups in both sexes in chickens during whole growth period of 42 d of age. Our results are concurrence with the previous studies of Ling and Jones (1995) and Yeganeh et al. (2005) in rats as well as in humans (Racette et al., 2010). Our results are not in agreement with the previous studies who concluded that the PS supplementation significantly decreased serum TG in hamsters (Chien et al., 2010; Misawa et al., 2002) in fish (Giman et al., 2003) and in humans (Sialvera et al., 2012). TG is blood lipids and derived from glycerol and three FA. TG in plasma is derived from fats eaten in foods or synthesize in the body from other energy sources like carbohydrates. In our present study, serum TG levels were no changed in all treatments. This might be due

to that the basic poultry diets in all treatments were composed on similar ingredients and providing the same amount of fat from these ingredients to all chickens throughout 42 d of feeding. However, the differences in results with other studies might be due to variations in the basic feed ingredients composition and its total fat content. Leptin has a major influence on energy balance. It is a mediator of long-term regulation of energy balance and inducing weight loss by suppressing food intake. Adipose tissues are the primary secretory source of leptin and its blood level depends on the amount of fat stored in adipocytes that is regulated by decreasing lipid synthesis and increasing fat mobilization. When leptin levels increase, food consumption decreases via modulation of hypothalamic neuropeptides. Our data of this present study showed that PS supplemented diets significantly improved serum leptin and insulin levels in both female and male broilers. Previous studies found that the PS supplementation had no effect on insulin concentrations in 21 d old rats (Furlan et al., 2012) in mice (Calpe-Berdiel et al., 2008) and in humans (Racette et al., 2010). The reason might be that the experimental diets supplemented with PS accelerate fat mobilization in chickens. It is also known that insulin promotes the synthesis of lipids and inhibits their degradation. Both males and females are susceptible to obesity. The frequency and health consequences differ between the sexes. Male and females differ in the patterns of fat deposition, fat mobilisation and utilisation of fat as a metabolic fuel (Power and Schulkin, 2008). Many of these differences may be due to reproductive system that is more nutritionally expensive for female than it is for male and the cause of the asymmetry in fat storage and in the utilization of fat as fuel. The results also

showed that PS supplemented diets had influenced on lipid metabolism in chickens. Obesity is the deposition of additional fat in the adipose tissue associated with over feeding, during a period of poor fat synthesis, an even greater decrease in the turnover and catabolism of adipose tissue fatty acids. It is also proved that lipid metabolism is different between female and male in humans (Blaak, 2001). However, a little information is available about lipid metabolism affected by PS supplementation in broiler chickens. Fatty acid synthase (FAS) plays a vital role in de nouo lipogenesis in mammals and birds. Fat deposition is determined by a complex balance between lipogenesis, lipolytic enzymes activities and fatty acids transport, as well as fatty acid utilization. It is well known that the lower lipogenic process was typically associated with reductions in activities of enzymes (Clarke, 1993; Mouroto et al., 1995; Katsurada et al., 1987). The data of present study showed that PS supplemented diets significantly decreased ($P < 0.05$) the activity of serum FAS levels in chickens. The diets supplemented with PS inhibited lipid deposition in liver. Hormone sensitive lipase (HSL) has opposite effect of mobilizing fatty acids from adipocytes into the bloodstream for lipolysis in adipocytes (Pashkov et al., 2005; Kokta et al., 2004; Gondret et al., 2000; Zubair and Leeson, 1994). The results of our present study suggested that poultry diets supplemented with PS 40mg/kg of diet was more effective in increasing serum HSL and FFA enzymes activities in female while PS 50mg/kg of diet increased serum HSL and FFA enzymes activities in male chickens and concluding that female broilers are more sensitive for PS diets as compared to male chickens. FFAs are involved in fat mobilization while leptin up-regulates the expression of the HSL enzyme

encoding gene, thus stimulating hydrolysis of triacylglycerols in adipose tissues. Some studies proved that the sterol could regulate fat enzyme activities (Schoonjans et al., 2000; Marinangeli et al., 2006; Rideout et al., 2010; Bennett et al., 1995), as well as the report of Thornton et al. (2011) suggested that dietary supplementation with PS reduced mass accumulation in obese mice. Sterol regulatory element binding transcription factor 1c (SREBP-1c), one of potential regulators that can directly stimulate the transcription of genes encoding FAS enzyme (Magana et al., 2000). The results of present study showed that the PS 40 and 50 mg/kg of diet decreased the mRNA expressions of both SREBP-1c and FAS enzymes in liver of female and male broiler chickens, respectively. These results are in consistent with the studies of Misawa (2012) who found that the oral feeding of antidiabetic PS (lophenol and cycloartanol) in rats significantly decreased FAS and SREBP-1c in mice. This suggested that PS may alter the expressions of various downstream enzymes genes that are involved in the synthesis of liver TG, and enhanced the lipolysis. Similarly, Rideout et al. (2010) found that the diets supplemented with PS increased hepatic lipogenic gene expressions of SREBP-1c (2. 4-fold) and FAS (6. 5-fold) as compared with control in mice. In the present study, supplementation with PS 40mg/kg of diet had more influenced in female broilers and down-regulated the gene expressions of hepatic enzymes FAS and SREBP-1c to inhibit lipid deposition in liver and up-regulated HSL enzyme activity to enhance lipolysis. These are in consistent with the change in serum leptin and FFA enzymes in female chickens. While, in male broiler chickens, PS 50mg/kg of diet significantly decreased the fat deposition by lowering the hepatic gene expressions of

FAS and SREBP-1c enzymes, as well as increased the fat mobilization, however, our data showed that insulin hormone level increased with PS supplemented diets in both female and male broilers. PS might improve the lipid metabolism, resulted in higher lipolysis than lipogenesis. Further experiments are required to exploit the exact mechanisms of these changes in chicken's fed diets supplemented with PS. In conclusion, PS supplemented diets had no significant influence on growth performance in AA broilers; however, female broiler chickens are more sensitive to PS diets than male chickens. The present study showed that poultry diets containing PS 40 and 50 mg/kg of diet might down-regulate fat deposition in female and male broiler chickens, respectively.