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Effects of Dietary Dihydropyridine Supplementation on Laying Performance and Fat Metabolism of Laying Hens

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ABSTRACT : The experiment was conducted to investigate the effects of dihydropyridine on laying performance and fat metabolism of laying hens. Five hundred and forty laying hens, 40 weeks old, were randomly allotted to three groups, each of which included four replicates of 45 hens. The groups were given a basal corn-soybean meal diet supplemented with 0, 150 mg/kg and 300 mg/kg dihydropyridine. Results showed that compared with the control group (0 mg/kg dihydropyridine), supplements of 150 and 300 mg/kg dihydropyridine increased egg production rate by 9.39% ($p<0.01$) and 12.97% ($p<0.01$), increased mean egg weight by 3% ($p>0.05$) and 4.8% ($p>0.05$), and improved feed efficiency by 9.54% ($p<0.05$) and 7.25% ($p<0.05$), respectively; The addition of 150 and 300 mg/kg dihydropyridine decreased percentage of abdominal fat by 35.4% ($p<0.05$) and 46.9% ($p<0.05$), decreased liver fat content by 32.4% ($p<0.05$) and 10.5% ($p<0.05$), increased HSL activity of abdominal fat by 39.64% ($p<0.05$) and 48.48% ($p<0.05$), increased HSL activity of liver by 9.4% ($p>0.05$) and 47.34% ($p<0.05$) and increased the content of cAMP in adenohypophysis by 14.67% ($p<0.05$) and 10.91% ($p<0.05$), respectively; The inclusion of 150 mg/kg dihydropyridine increased liver superoxide dismutase activity by 69.61% ($p<0.05$), and increased hepatic apoB concentration by 53.96% ($p<0.05$); The supplementation of 150 or 300 mg/kg dihydropyridine decreased malondialdehyde concentration of hepatic mitochondria by 30.90% ($p<0.01$) and 10.39% ($p<0.05$), respectively; Supplemented dihydropyridine had no significant effects on TG, Ch HDL-C and VLDL-C concentrations in serum; addition of 150 or 300 mg/kg dihydropyridine increased T_3 levels in serum by 15.34% ($p<0.05$) and 11.88% ($p<0.05$) and decreased insulin concentration by 40.44% ($p<0.05$) and 54.37% ($p<0.05$), respectively. The results demonstrated that adding dihydropyridine had the tendency of improving very low density lipoprotein receptor (VLDLR) content in the ovary. It was concluded that dihydropyridine could improve laying performance and regulate the fat metabolism of laying hens and that 150 mg/kg dihydropyridine is the optimum dose for laying birds in practical conditions. **Key Words :** Dihydropyridine, Laying Performance, Fat Metabolism, Laying Hens, TG (Triglycerides), Ch (Cholesterol), HDL-C (High Density Lipoprotein-Cholesterol), VLDL-C (Very Low Density Lipoprotein-Cholesterol), HSL (Hormone-sensitive Triglyceride Lipase), SOD (Superoxide Dismutase), MDA (Malondialdehyde), T3 (Triiodothyronine), T4 (Thyroxine), cAMP (Cyclic adenosine monophosphate), VTGR (Vitellogenin receptor)

INTRODUCTION

The liver is the major organ to synthesize yolk materials in laying fowl (Legrand, 1987). Most substances are synthesized and transported from the liver in the form of very low density lipoprotein (VLDL) to the follicle as precursors of egg yolk. Under normal physiological status, the fat content of liver in laying hens will gradually increase with time during the middle- and late-laying stage. When a certain amount of fat has accumulated, the normal function of liver and the development and maturation of ovarian follicle will be affected (Banerjee, 1984). Hens at peak laying are especially prone to disorders of lipid metabolism due to intensive body metabolism, which causes the synthesis and transport of VLDL to be blocked. Because the synthesized fat cannot be transported fast enough, excessive triglyceride accumulates in liver. Meanwhile, under the stress of cage-breeding and continuous ovulation and laying, the laying fowl body produces large quantities of free radicals. However, the ability to eliminate free radicals decreases gradually over time. Free radicals act on hepatic organelle and intracellular macromolecules, destroying the morphology and function of hepatic membrane and submembrane. Laying hens tend to exhibit fatty liver haemorrhagic syndrome (FLHS) due to the damage caused by free radicals and excessive fat in the liver. The effect of type and level of certain fatty acids may play an important role in the occurrence of the disorder. There is a correlation between delta-9 desaturase enzyme activity and secretion of

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VLDL from the liver (Rahim, 2005). This disease is very common among the cage-breeding laying hens and its prevalence in the poultry industry of many countries (Ubosi, 1990) constitutes a hidden threat to modern intensive poultry production because of the potentially serious economic loss. Dimitrov (1980) reported that mortality due to liver obesity syndrome varies within the range of 3.1 to 3.7% for the entire period of exploitation. Rahim (2005) indicated that the first sign of fatty liver syndrome is often an increase in mortality in the flock. Many studies on FLHS of laying fowl have been carried out, but there is still no effective method to resolve it.

Dihydropyridine is a new multifunctional antioxidant in food (Kourimska, 1993; Smagin, 1998; Tirzitis, 1999; Panek, 2000; Tirzitis, 2001). Dihydropyridine was used as an antioxidant in feeding of broiler ducks (Bakutis, 1984) and had antioxidant and immunological activity in the feeding of chickens (Val'-dman, 1990). Dihydropyridine improved the health of the cows and their calves, increased the yield of milk, improved the quality of milk and colostrum and increased cow fertility (Sutkevicius, 1984); it also reduced body weight loss in young fattening cattle during transport to the meat-packing plant (Spruzh, 1991). Dihydropyridine improved daily weight gain and feed conversion efficiency of growing pigs and decreased fat thickness (Wu, 1999) and regulated fat metabolism in humans (Zhang, 2002). However there is still no report on its function in regulating fat metabolism in laying hens. Ttherefore this research studied effects of dihydropyridine on laying performance and fat metabolism of laying hens to provide clues for developing a new feed additive which can promote production and reduce the incidence of fatty liver haemorrhagic syndrome (FLHS).

MATERIALS AND METHODS

Experimental Design and Birds

All procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang University. A total of 540 healthy, 300 d old laying hens were randomly distributed to 3 groups. Each group had four replicates each comprising 45 laying hens. These hens were given corn-soybean meal diets supplemented with 0 (the control), 150 mg/kg and 300 mg/kg dihydropyridine. The dihydropyridine was provided by Sunpu Biochem.Tech.Co., Ltd, Beijing, China. The hens were kept in three-layer complete ladder cages with feed and water supplied *ad libitum*. During the whole experimental period (including 7 d of pre-experiment and 90 d of experiment), a 16-h photoperiod was maintained. Feed intake, number of eggs and total egg weight were recorded daily.

At the end of the experiment, two birds from each replicate were euthanized under anaesthesia and exsanguinated after a 12 h fast and *ad libitum* access to water. The carcass was weighed after viscera were removed, and abdominal fat was dissected and weighed. The abdominal fat percentage (AFP) was calculated as: AFP = abdominal fat weight/carcass weight×100%.

Blood samples were obtained via vena cava puncture and fresh blood was poured into a vessel. After being separated out naturally, serum was transferred into an Eppendorf centrifuge tube (10 ml), centrifuged for 10 min (5,000×g), and then kept at -30°C until analysis.

Liver and abdominal fat samples were taken from the same carcass, frozen in liquid nitrogen, then kept at -30°C until analysis.

Ovarian follicles with a diameter of 2-15 mm were collected and washed with Tris-HCl buffer at pH 8.0 (containing 20 mmol/L Tris-HCl, 1 mmol/L$CaCl_2$, 150 mmol/L PM SF, 2 mmol/L Leupetin), then stored under -20°C until analysis.

Experimental parameters measured

Determination of hepatic fat percentage : Liver samples were homogenized and dried to a constant weight at 105°C. A dried sample (2 g) was extracted with a chloroform: ether mixture (5:1) in a soxhlet apparatus for 8 h, and the extracted sample was dried to constant weight at 105°C. Fat weight was calculated by subtracting sample weight after extraction from sample weight before extraction and hepatic fat percentage as: fat weight/dried liver weight ×100%.

Determination of SOD activity and apoA, apoB and MDA content in liver : The liver tissue was homogenized with 0.9% sodium chloride solution to make a 10% hepatic mixture, which was then centrifugated at 3,000 r/min for 10-15 min. The upper pellucid liquid was taken for parameter determinations. The SOD activity was determined by colorimetry according to the assay kit provided by Nanjing Jiancheng Bioengineering Institute. ApoA, apoB and MDA content were determined by UV-2,000 spectrophotometer (UNICO Instruments Co., Ltd., Shanghai, China) using analysis kits provided by Ningbo City Bio-chemical Reagent Factory and Nanjing Jiancheng Bioengineering Institute, China.

Determination of serum biochemical parameters related to fat metabolism : Serum triglycerides (TG), cholesterol (Ch), high density lipoprotein-cholesterol (HDL-C), and very low density lipoprotein-cholesterol (VLDL-C) were measured by ERBA CHEM-5 semi-automatic biochemical analyzer using analysis kits provided by Ningbo City Bio-chemical Reagent Factory., China.

Determination of activity of hormone-sensitive triglyceride lipase (HSL) in liver and abdominal fat

HSL activity was measured using the method reported by Shih (1995). The fat was minced and homogenized in 10

Table 1. Ingredients and nutrient composition of diets

Ingredient	Composition (%)	Nutrient	Level
Corn	65	DE	12.43 MJ/kg
Soybean meal	17.7	CP	15.44%
Rapeseed meal	4	Ca	3.25%
Bran	2	Available P	0.25%
Calcium hydrogen Phosphate	0.63	Met+cys	0.58%
Stone meal	7.75	Thr	0.52%
Salt	0.3	Lys	0.85%
Fish meal	0.8		
Met	0.16		
Mineral and vitamin premix[1]	1		

The premix was provided by the Feed Institute of Zhejiang University.

volumes of pH 7.0 medium containing 0.25 M sucrose, 1mM EDTA, 4 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 mM dithiothreitol then centrifuged at 105,000 g for 45 min at 4°C. The superficial'fat cake' was removed and the clear supernatant decanted and used for the assay of HSL activity based on the standard assay procedure of Nilsson-Ehle and Schotz. The assay substrate was prepared immediately before use to give a final concentration of each reagent in the 200 μl assay volume as follows: 100 mmol/L Tris HCl, pH 7.0; 5 g/L of BSA (fatty acid-free, fraction V), and 4.58 mmol/L triolein as substrate. HSL activity units were defined as: one HSL activity unit is the concentration of enzyme that hydrolyzes 1 umol olive oil in one minute at 37°C.

Determination of hormone concentrations in serum : Concentrations of triiodothyronine (T3), thyroxine (T4) and insulin (Ins) in serum were measured using an RIA kit (Beijing North Immuological Institute, China) and a gamma-counter (Packard 8500, USA).

Determination of content of vitellogenin receptor (VTGR) : VTGR content was measured according to the method of George (1987).

Statistical analysis

All the data were analyzed statistically using the general linear model procedure (SAS Institute, 1989) and the treatment means were separated by Duncan's multiple range test.

RESULTS

Effects of dihydropyridine on laying performance

Results for egg production rate, mean egg weight, feed intake and feed efficiency are presented in Table 2. Compared with the control group (0 mg/kg dihydropyridine), supplements of 150 or 300 mg/kg dihydropyridine increased egg production rate by 9.4% ($p<0.01$) and 13.0% ($p<0.01$), increased mean egg weight by 3% ($p>0.05$) and 4.8% ($p>0.05$), and improved feed efficiency by 9.5% ($p<0.05$) and 7.3% ($p<0.05$), respectively. There were no significant differences in laying rate and feed:egg weight between 150 and 300 mg/kg dietary dihydropyridine.

Effects of dihydropyridine on fat metabolism of laying hens

Compared with the control group (Table 3), 150 mg/kg dihydropyridine supplementation decreased hepatic fat percentage and abdominal fat percentage by 32.4% ($p<0.05$) and 35.4% ($p>0.05$), respectively, and 300 mg/kg dihydropyridine supplementation decreased hepatic fat percentage and abdominal fat percentage by 10.5%

Table 2. Effects of dihydropyridine on laying performance

Item	Dihydropyridine (mg/kg)			SEM
	0	150	300	
Egg production rate (%)	64.78±3.79^b	70.86±1.68^a	73.18±2.24^a	1.36
Mean egg weight (g)	50.12±3.28	51.99±0.84	52.53±1.30	1.05
Daily feed intake (g)	88.54±9.56	86.20±6.35	90.40±8.65	4.15
Feed/Egg weight	2.62±0.33^a	2.37±0.20^b	2.43±0.22^b	0.13

$^{a, b}$ Means in a row with different superscripts are significant different ($p<0.05$).

Table 3. Effects of dihydropyridine on HFP, AFP, HSL activity, cAMP content, SOD activity and MDA content

Item	Dihydropyridine (mg/kg)			SEM
	0	150	300	
Hepatic fat percentage (%)	33.44±8.56^a	22.59±1.62^b	29.92±6.87^a	3.20
Abdominal fat percentage (%)	4.86±c3.54^b	3.14±2.03^b	2.58±1.42^a	1.25
HSL activity in liver (IU)	39.64±1.83^b	43.32±1.93^a	42.58±0.96^a	0.82
HSL activity in abdominal fat (IU)	21.64±1.09^b	30.18±1.37^a	32.13±1.42^a	0.65
cAMP in adenohypophysis (pm/g)	238.22±27.16^b	273.16±25.30^a	264.21±26.13^a	13.10
SOD U/mg prot	22.74±3.34^b	38.57±2.46^a	22.21±7.49^b	2.47
MDA in mitochondria (nmol/L)	1.52±0.18^a	1.05±0.28^c	1.37±0.65^b	0.21

$^{a-c}$ Means in a row with different superscripts are significant different ($p<0.05$).

Table 4. Effects of dihydropyridine on apolipoprotein content in liver, on serum biochemical parameters and on vitellogenin receptor (VTGR) content of oocytes

Item	Dihydropyridine (mg/kg)			SEM
	0	150	300	
ApoB mg/100 mg prot	30.19±7.55[b]	46.48±5.96[a]	27.44±3.41[b]	2.95
ApoAmg/100 mgprot	18.99±2.36	17.61±1.83	20.18±3.21	1.27
Cholesterol (mg/dl)	118.73±52.25	114.01±19.32	125.59±50.68	21.74
TG (mmol/L)	7.99±2.58	8.06±6.20	9.99±5.69	2.54
VLDL-C (mmol/L)	1.86±0.52	1.08±0.56	1.32±1.13	0.39
HDL-C (mg/dl)	129.16±32.55	141.26±17.68	126.27±47.91	17.48
T3 (ng/ml)	2.02±0.31[b]	2.33±0.37[a]	2.26±0.45[a]	0.19
T4 (ng/ml)	23.80±1.56	23.03±4.96	23.14±4.51	1.99
Insulin (ng/ml)	9.62±1.55[b]	5.73±1.27[a]	4.39±1.75[a]	0.77
VTGR (mg/g prot)	0.27±0.01	0.34±0.03	0.33±0.02	0.01

[a, b] Means in a row with different superscripts are significant different ($p<0.05$).

($p>0.05$) and 46.9% ($p<0.05$), respectively; HSL activity in abdominal fat and liver increased by 39.5% ($p<0.05$) and 9.4% ($p>0.05$), respectively, in the 150 mg/kg dihydropyridine group and increased by 48.5% ($p<0.05$) and 47.3% ($p<0.05$), respectively, in the 300 mg/kg group; 150 and 300 mg/kg dihydropyridine decreased cAMP content in adenohypophysis of laying hens by 14.7% ($p<0.05$) and 10.9% ($p<0.05$).respectively.

Compared with the control group (Table 3), addition of 150 mg/kg dihydropyridine increased SOD activity of liver by 69.6% ($p<0.01$); 300 mg/kg dihydropyridine had no significant effect on SOD activity; 150 and 300 mg/kg dihydropyridine supplementation decreased mitochondrial MDA concentration in liver by 30.9% ($p<0.01$) and 10.4% ($p<0.05$), respectively.

Results for apolipoprotein content in liver and serum biochemical parameters are presented in Table 4. Compared with the control group, 150 mg/kg dihydropyridine addition increased apoB content in liver by 54.0% ($p<0.05$), 300 mg/kg dihydropyridine had no significant difference on apoB content; dihydropyridine supplement had no significant effects on content of TG, Ch, VLDL-C and HDL-C in serum.

Effects of dihydropyridine on T_3, T_4 and insulin level in serum are present in Table 4; 150 and 300 mg/kg dihydropyridine increased T_3 levels in serum by 15.3% ($p<0.05$) and 11.9% ($p<0.05$), respectively, decreased insulin levels by 40.4% ($p<0.01$) and 54.4% ($p<0.01$) respectively, but dihydropyridine supplements had no significant effect on T_4 levels in serum.

Effects of dihydropyridine on VTGR content of developing oocyte membrane are presented in Table 4; 150 and 300 mg/kg of dihydropyridine supplement increased VTGR content in vitelline membrane of laying hens by 24.5% ($p>0.05$) and 21.5% ($p>0.05$), respectively, which indicated a trend for increasing VTGR content with dihydropyridine supplementation.

DISCUSSION

Effects of dihydropyridine on laying performance

This study showed dihydropyridine significantly improved laying performance in laying hens. Chen (1993) reported that 150 mg/kg dihydropyridine increased egg production rate by 12.9% and improved feed efficiency by 13.5%. Zou (1998) pointed out that 150 mg/kg dihydropyridine increased egg production rate by 11.1%. and improved feed efficiency by 10.4%. These results all indicated that dihydropyridine could promote laying performance in laying hens.

Effects of dihydropyridine on fat metabolism of laying hens

This study showed that dihydropyridine supplementation significantly reduced abdominal fat percentage at the late laying stage. Borchni (1991) and Zhang (2002) reported that dihydropyridine decreased serum lipid concentration and reduced serum triglyceride concentration through restraining synthesis and organic absorption of triglyceride and cholesterol. In the present study dihydropyridine significantly increased HSL activity in abdominal fat of laying hens. HSL is the key enzyme in adipocyte lipolysis (Lan, 1996; Diaz, 1999) which can hydrolyze triglyceride to glycerol and fatty acids to meet body requirements. HSL activity is affected by cAMP directly and this study indicated that dihydropyridine improved cAMP level in adenohypophysis of laying hens. cAMP acts as a secondary messenger to activate protein kinase which activates HSL by phosphorylation to make it functional in lipid hydrolysis.

The present study indicated that dihydropyridine significantly increased SOD activity of serum and liver in laying hens and significantly decreased MDA content in liver. This observation is consistent with the results of Sniedze (1977) which showed that dihydropyridine had an antioxidant function in animals and restrained the oxidation of lipid compounds. SOD protects important organs from attack by free radicals and maintains their normal physiological function by disposing of excessive free radical. Paradis (1997) reported that dihydropyridine combined with terminal oxidase cytochrome P450 to form a complex which significantly restrained activity of NADPH-cytochrome-c reductase, restrained NADPH production and then reduced lipid superoxidation.

Results of this study also showed that dihydropyridine significantly improved the level of apolipoprotein B in the liver of laying hens. ApoB 100 is one of the components of VLDL, which transports triglycerides out of liver. ApoB 100 is the most important component of VLDL which is synthesized by liver. Yi (2000) showed by immune electron microscopy that fat was joined to the apoB chain to form VLDL which then transports endogenetic fat to extrahepatic tissues. Lien (1999) pointed out that increased synthesis of apoB could reduce hepatic fat accumulation and accelerate transport of fat out of liver. Schneider (1996) reported that in fowl vitellogenin and VLDL were the primary precursors synthesized by liver. Increased apoB level can promote the transport of VTG together with VLDL and improve the content of lipoprotein in yolk (Gordon, 1994).

This experiment showed that dihydropyridine supplementation tends to increase the content of VTGR in the vitelline membrane of laying hens, which may be the mechanism by which dihydropyridine improved the egg laying rate and egg weight. VTGR is an essential receptor in fowl, which is only expressed in oocytes of female birds and has an important physiological function in regulation of oogenesis and development. Barber (1991), Ninpf (1987) and Schneider (1996) found that the VTGR of the oocyte membrane combined with two important lipoproteins (VLDL and VTG) of the yolk and thereby regulated VLDL and VTG entry into the oocyte by endocytosis. Maclachlan (1994) reported that oocyte VTGR not only transported the main lipid into yolk, but also transported nonlipoprotein, and therefore the content of vitellogenin receptor directly affected egg production rate, mean egg weight and fat metabolism in laying hens. Increased content of VTGR can promote fat transportation out of the liver, improve egg laying rate and decrease fat accumulation in liver.

Dihydropyridine could regulate incretion in the body (Zou, 1999). The present results demonstrated that dihydropyridine increased T_3 level in serum and simultaneously decreased insulin level, which agrees with observations by Wu (1999) in pigs. T_3 promotes fat mobilization and enzyme hydrolyzation, and insulin has the reverse effects. Valcavi (1997) and Tashi (1998) found that there was a positive correlation between thyroxine and leptin levels and thyroxine stimulated the secretion of leptin. Leptin is a protein secreted by adipocytes which maintains the relative stability of body fat by regulating energy metabolism. Leptin absence will induce increased insulin level in serum and stimulate hepatic fatty acid synthesis, as already shown in mice with leptin absence. In mice whose leptin receptor genes were knocked-out, symptoms of insulin resistance and fatty liver were still present even if serum leptin level increased. So it is speculated that dihydropyridine can regulate fat metabolism of laying hens through regulating the secretion of leptin in adipocytes.

Dihydropyridine affected the secretion of T3 and insulin, affected the activity of SOD and HSL, affected VLDL concentration and affected the hepatic and abdominal fat percentages, which showed that dihydropyridine had effects on the synthesis, transportation and deposition of fat in liver.

IMPLICATION

This study indicated dihydropyridine coud improve laying performance and decrease hepatic fat percentage and abdominal fat percentage by affecting fat metabolism in laying hens. 150 mg/kg dihydropyridine is the optimum dose for laying birds in practical conditions.

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