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Performance and Serum Hepatic Enzymes of Hy-Line W-36 Laying Hens Intoxicated with Dietary Carbon Tetrachloride

Hadavi A, Kermanshahi H, Nassiri Moghaddam H & Golian A

Department of Animal Science, Faculty of Agricultural, Ferdowsi University of Mashhad, Mashhad, Iran

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Corresponding author
Hassan Kermanshahi
kermansh@um.ac.ir

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Abstract

An experiment was conducted to study the effects of carbon tetrachloride (CCl_4) on post-peak performance and serum enzymes of Hy-Line W-36 laying hens from 32-36 weeks of age. The experiment was carried out with a total of 192 laying hens in a completely randomized block design. During the experiment laying hens were allocated to 4 groups consisted of T_1) no CCl_4 as control diet, T_2, T_3 and T_4) control diet supplemented with 1, 3 and 5 mL CCl_4/100 g diet, respectively. Each experimental group was divided into 6 blocks of 8 hens each. Egg production, cracked egg percentage and feed intake were recorded weekly. Blood samples were taken from wing veins of hens at the middle and end of the experiment to measure serum hepatic enzymes of alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase. Data showed that in comparison with the control group, the inclusion of CCl_4 to the diets had no significant effect on performance parameters. However, by increasing the level of CCl_4, egg production was linearly decreased and feed intake was linearly increased ($P < 0.05$). The effect of CCl_4 on cracked eggs was significant and this effect was linearly increased ($P < 0.05$). Dietary supplementation of 3 and 5 mL CCl_4 elevated the serum concentration of hepatic enzymes of alkaline phosphatase, aspartate aminotransferase and alanine aminotransferase, linearly ($P < 0.0001$). In conclusion, the dietary supplementation of CCl_4 has the ability to decrease the performance and egg quality. CCl_4 is also a potent hepatic toxicity inducer and may damage liver hepatocytes. Therefore, the level of 3 mL CCl_4 was assigned as the one had the maximum negative effect on serum hepatic enzymes concentration (maximum liver damage) alongside the minimum negative effect on laying hen performance for further studies.

Introduction

In the modern poultry production, the birds are exposed to different stressful factors. Oxidative stresses produce free radicals suppressing the bird performance by disordering the body homeostasis. The liver is the best tissue to evaluate the oxidant-induced oxidative stresses. CCl_4 is an important service today as a toxic agent model to induce oxidative stress on animals leading to liver cirrhosis and fibrosis (Tsukamoto *et al.*, 1990).

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The liver is the principal site of detoxification of CCl_4 and the increase in serum concentration of alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) is caused by hepatocyte injury, resulting from necrosis or changes in cell membrane permeability, and can be attributed to the liver dysfunction (Tenant, 1997). Liver cell injury induced by CCl_4 involves initially the conversion of CCl_4 by the cytochromes P-450 of hepatic cells to an active metabolite (trichloromethyl, CCl_3^-), which is a highly reactive free radical. Then trichloromethyl free radical reacts with oxygen and is converted to proxy trichloromethyl (CCl_3OO). Proxy trichloromethyl attacks the endoplasmic reticulum membrane and causes lipid peroxidation, loss of cellular calcium, decreased protein synthesis, increased liver enzymes, and eventual destruction of the liver cells (Panovska *et al.,* 2007). With the loss of cell membrane components, enzymes leak so that intracellular fluid increases (Weber *et al.,* 2003). Khorramshahi *et al.* (2014) demonstrated that CCl_4 injection caused liver toxicity in Japanese quails and damaged liver cells by causing the formation of bubble-like structures in the liver tissue, shrinking of the sinusoid space and inflammation in parts of the liver parenchyma as well as an abnormality of the hepatic artery and bile duct in liver tissue. The similar liver damage by CCl_4 was reported on broilers (Sonkusale *et al.,* 2011; Nateghi *et al.,* 2013) and mice (Robjohns, 2009).

The effects of CCl_4 in such mammals as mice, rats, rabbits and guinea pigs have been described in great detail, but there are limited researches of CCl_4 effects on non-mammalian species, such as the laying hens. Thus, the aim of this experiment was to investigate the effects of dietary supplementation of different levels of CCl_4 on laying hens performance to find the level of CCl_4 which had the maximum increasing effect on serum hepatic enzymes concentration alongside the minimum negative effect on laying hens performance. This level will be used for later studies when CCl_4 is needed to induce hepatotoxicity in laying hens.

Materials and Methods

Laying hens, diets and management

The experimental protocols were approved by the Animal Care Committee of Ferdowsi University of Mashhad, Iran. During the experiment (32-36 weeks of age), a total number of 192 Hy-Line W-36 laying hens with the uniform body weights were allocated to 4 experimental groups consisted of T_1: no CCl_4 as control diet, T_2, T_3 and T_4: control diet supplemented with 1, 3 and 5 mL CCl_4/100 g diet (99.9% purity, Merck, Germany), respectively, in a completely randomized block design to induce chronic damage in the liver. Each experimental group was divided into 6 blocks consisting of 8 hens each (8 birds/2-cage unit per block). The laying hens were fed to match the requirements recommended by the Hy-Line W-36 recommendations (Hy-Line International, 2005). The ingredients and chemical composition of the basal diet are shown in Table 1. Laying hens were housed in standard battery cages with dimensions of 40 × 40 cm, equaling 1600 cm^2 of floor space. With 4 hens per cage, each bird had approximately 400 cm^2 of floor space. Each cage was equipped with a feeding trough and nipple drinkers and hens had free access to feed and water. During the study, the hens received a constant lighting regimen of 16 hrs light: 8 hrs darkness and the temperature was set at 21°C.

Table 1. The ingredients and chemical composition of the basal diet fed to laying hens

Ingredients	g/kg
Corn	500.0
Wheat	181.0
Soybean meal, 44% protein	190.0
Soya oil	10.0
$CaCo_3$	94.0
Dicalcium phosphate	15.0
Salt	3.0
Vitamin and mineral premix[1]	5.0
DL-Methionine	2.0
Calculated analysis (%, unless otherwise noted)	
ME, kcal/kg	2745
CP	15.30
Met	0.34
Met + Cys	0.60
Lys	0.66
Thr	0.50
Ava. P	0.40
Ca	3.90
Na	0.18

[1]Supplied per kilogram of diet: vitamin A, 10000 IU; vitamin D_3, 9790 IU; vitamin E, 121 IU; vitamin K_2, 2 mg; vitamin B_{12}, 0.02 mg; thiamin, 4 mg; riboflavin, 4.4 mg; niacin, 22 mg; pyridoxine, 4 mg; biotin, 0.03 mg; folic acid, 1 mg; Ca-pantothenate, 40 mg; choline chloride, 840 mg; ethoxyquin, 0.125 mg; Zn, 65 mg; Mn, 75 mg; Cu, 6 mg; Se, 0.2 mg; I, 1 mg; Fe, 75 mg.

Sample collection and measurements

Egg production and cracked eggs were recorded daily and expressed as weekly basis. Feed intake for each replicate was determined from the difference of feed offered and feed weighed back during each week.

Blood samples were taken from the wing veins of the hens at the middle and end of the experiment (32 and 36 weeks, respectively) to measure serum concentration of hepatic enzymes of ALP, ALT and AST. Blood samples were transferred to tubes and kept at room temperature to clot. Blood serum samples were then centrifuged at 2,000 × g for 15 min at 4°C to separate the impurity of the samples. The separated serum samples were kept at -20°C for later measurement of hepatic enzymes of ALP, ALT and AST by an autoanalyzer (Selectra E vital scientific, Dieren, Netherlands).

Statistical analysis

Prior to analysis, all of the percentage data were normalized by subjecting to arc sine transformation. As the cage represented the experimental unit, the effects of dietary supplementation of CCl_4 on laying hens performance was statistically analyzed in a completely randomized block design using the GLM procedure of SAS (2001). Treatment means were compared using Tukey's multiple range test. A value of $P < 0.05$ was considered significant. Orthogonal polynomial contrasts were used to test the linear, quadratic and cubic effects of the increasing levels of CCl_4 supplementation.

Results

The effects of dietary supplementation of CCl_4 on laying hens' egg production, cracked egg percentage and feed intake are presented in Tables 2, 3 and 4, respectively. Data showed that in comparison with the control group, although the inclusion of CCl_4 to the diets had no significant effect on performance parameters, but 5 mL CCl_4 increased ($P < 0.05$) total cracked eggs. By increasing the level of CCl_4, egg production was linearly decreased ($P < 0.05$) while feed intake and cracked eggs were linearly increased ($P < 0.05$).

Table 2. Effects of dietary supplementation of carbon tetrachloride (CCl_4) on egg production of laying hens from 32-36 weeks of age

Treatments	Egg production (%)				
	wk33	wk34	wk35	wk36	wk32-36
Control	86.2	89. 6	89.0	89.0	88.6
1 mL CCl_4/100 g diet	83.4	87.5	86.9	87.5	86.6
3 mL CCl_4/100 g diet	82.6	85.4	86.9	84.0	85.0
5 mL CCl_4/100 g diet	81.7	80.6	86.1	82.4	83.2
SEM	1.67	3.73	2.56	3.46	1.77
Source of variation (P-values*)*					
Treat	0.283	0.393	0.870	0.531	0.209
Linear	0.071	0.102	0.457	0.155	0.039
Quadratic	0.576	0.717	0.810	0.981	0.950
Cubic	0.785	0.871	0.802	0.816	0.945
Block	0.496	0.565	0.245	0.698	0.629

[a,b]Means in each column with different superscripts are significantly different ($P < 0.05$).

Table 3. Effects of dietary supplementation of carbon tetrachloride (CCl_4) on cracked eggs of laying hens from 32-36 weeks of age

Treatments	Cracked Eggs (%)				
	wk33	wk34	wk35	wk36	wk32-36
Control	2.38	7.14	5.55	0	3.77[b]
1 mL CCl4/100 g diet	3.33	7.50	3.33	3.33	4.37[b]
3 mL CCl4/100 g diet	7.50	8.93	8.50	4.76	7.42[ab]
5 mL CCl4/100 g diet	11.27	12.10	12.30	9.02	11.17[a]
SEM	3.31	3.26	3.70	2.78	1.61
Source of variation (P-values*)*					
Treat	0.249	0.701	0.383	0.190	0.020
Linear	0.055	0.280	0.147	0.037	0.003
Quadratic	0.676	0.671	0.430	0.796	0.347
Cubic	0.810	0.964	0.603	0.709	0.813
Block	0.999	0.253	0.790	0.632	0.321

[a,b]Means in each column with different superscripts are significantly different ($P < 0.05$).

Table 4. Effects of dietary supplementation of carbon tetrachloride (CCl_4) on feed intake of laying hens from 32-36 weeks of age

Treatments	Feed intake (g/hen/day)				
	wk33	wk34	wk35	wk36	wk32-36
Control	96.8	105.3	100.2	101.2	100.8
1 mL CCl_4/100g diet	96.8	105.6	100.9	102.9	101.7
3 mL CCl_4/100g diet	97.4	106.4	102.6	104.7	103.1
5 mL CCl_4/100g diet	98.7	107.9	104.2	104.4	104.0
SEM	2.17	2.49	2.01	2.38	0.99
Source of variation (*P*-values)					
Treat	0.915	0.880	0.500	0.724	0.149
Linear	0.525	0.449	0.141	0.305	0.026
Quadratic	0.771	0.812	0.834	0.685	0.982
Cubic	0.989	0.975	0.908	0.841	0.824
Block	0.541	0.617	0.718	0.363	0.341

[a,b]Means in each column with different superscripts are significantly different ($P < 0.05$).

The effects of dietary supplementation of CCl_4 on laying hens serum concentration of hepatic enzymes are presented in Table 5. Dietary supplementation of 3 and 5 mL CCl_4 linearly elevated ($P < 0.05$) the serum concentration of hepatic enzymes of ALP, AST and ALT, while 1 mL CCl_4 had no significant effect on the serum concentration of enzymes.

Table 5. Effects of dietary supplementation of carbon tetrachloride (CCl_4) on serum concentration of hepatic enzymes in laying hens at 34 and 36 weeks of age

Treatments	ALP[1]		ALT[2]		AST[3]	
	wk34	wk36	Wk32	Wk36	Wk32	Wk36
Control	963.0[b]	2064.8[b]	4.66[b]	3.33[b]	129.6[b]	135.3[b]
1 mL/100 g CCl_4	1035.0[b]	2077.3[b]	5.00[b]	3.50[b]	133.3[b]	139.6[b]
3 mL/100 g CCl_4	1225.5[a]	3347.3[a]	7.00[a]	5.16[a]	169.3[a]	176.0[a]
5 mL/100 g CCl_4	1256.5[a]	3160.2[a]	6.66[a]	5.00[a]	165.1[a]	172.1[a]
SEM	35.93	174.84	0.304	0.360	1.661	5.020
Source of variation (*P*-values)						
Treat	0.0001	0.0001	0.0001	0.003	0.0001	0.0001
Linear	0.0001	0.001	0.001	0.0009	0.0001	0.0001
Quadratic	0.172	0.576	0.290	0.650	0.032	0.428
Cubic	0.024	0.003	0.010	0.056	0.0001	0.005
Block	0.338	0.663	0.099	0.990	0.977	0.979

[a-b]Means in each column with different superscripts are significantly different ($P < 0.05$).
[1]Alkaline phosphatase; [2]Alanine aminotransferase; [3]Aspartate aminotransferase.

Discussion

This study indicated that linear dose response of CCl_4 on egg production, feed intake and cracked eggs was significant. By increasing the level of CCl_4 from 1 to 3 and 5 mL/100 g diet, egg production was decreased and feed intake and cracked eggs were increased. It has been shown that the toxins destruct the epithelial cells of the intestinal wall and change the intestinal ecosystem leading to the suppressed performance in laying hens (Applegate *et al.*, 2009).

In this study, dietary supplementation of CCl_4 significantly changed the serum concentration of hepatic enzymes in laying hens. These results were in agreement with those of Khorramshahi *et al.* (2014) who reported that the Japanese quails treated with CCl_4 intraperitoneally showed an increment ($P < 0.05$) in the serum concentration of hepatic enzymes of ALP, AST and ALT. Sonkusale *et al.* (2011) and Nateghi *et al.* (2013) showed that inclusion of CCl_4 to broilers diets increased hepatic enzymes levels. ALT is present in the cytoplasm of liver cells while AST exit in the mitochondria. ALP is also present in the liver. CCl_4-induced liver damage stimulates defective hepatic metabolic function, resulting in increased serum concentration of hepatic enzymes of ALP, AST and ALT (Mandrekar and Szabo, 2009) which are the main indicators of liver damage. Mansour (2000) and Ali *et al.* (2010) reported that CCl_4-induced hepatotoxicity in mice manifested biochemically by significant elevation of activities

of liver functions, such as ALT and AST.

Previous studies have shown that poultry, unlike other laboratory animals such as mice, are resistant to necrogenic effects of CCl_4-induction. This lack of sensitivity in poultry is for this reason that their liver does not activate CCl_4 to active metabolites, including free radicals of CCl_3^-. This low capacity for CCl_4 activation might be due to a lower content of cytochrome P-450 in the liver of poultry compared with more susceptible species such as mice. Cytochrome P-450 plays a key role in CCl_4 activation and other toxins to active metabolites in hepatocytes. These metabolites, in turn, react with lipids and proteins and eventually cause liver damage (Slater, 1966; Diaz Gomez *et al.*, 1975). However, new researches reported higher sensitivity to the necrogenic effects of CCl_4 in broilers and Japanese quails than those of previous studies (Nateghi, 2011; Sonkusale *et al.*, 2011; Samadi *et al.*, 2015). This is probably due to the nowadays genetic manipulation and selection of the mentioned birds resulting in the lowered immunity leads to the less resistance to CCl_4.

Conclusion

The dietary supplementation of CCl_4 tended to suppress performance and elevated secretion of hepatic enzymes of ALP, AST and ALT into the blood of laying hens. The level of 3 mL CCl_4 was assigned as the one that had the maximum increasing effect on serum hepatic enzymes concentration (the maximum liver damage) alongside the minimum negative effect on laying hen performance. Increased serum concentration of hepatic enzymes is the main indicator of liver damage and can be used to assess the hepatoprotective effects of different additives in both *in vivo* and *in vitro* conditions.

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