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Effect of Malic Acid on Some Serum Metabolite and Hepatic Enzymes in Chickens

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Abstract: Two experiments were conducted to determine the effects of the malic acid on chicken liver parameters and some serum metabolites. First study was performed with male egg type chickens (2 to 21 d of age) which received four levels of malic acid via drinking water. Malic acid was added to the water and offered to chicken freely from first to end of experiment with constant concentration in both experiments. The treatments were zero (as a control), 0.05, 0.10, and 0.15 percent of malic acid which dissolved in water and given to them in waterer pan. The chicks were slaughter on 21 days old and triacylglycerols (TAG) and cholesterol were measured on blood serum along with liver enzyme including malate dehydrogenase (MDH) and isocitrate dehydrogenase (IDH). In second experiment broiler chicken (male and female from 1 to 56 d of age) was evaluated for same parameters on same treatments as a mentioned for the first experiment. No significant difference (P> 0.05) was observed between treatments for weight gain in both experiments. Difference between treatments in relation to serum cholesterol and triacylglycerols was observed in both experiments (P<0.05) but with different patterns. In the first experiment, liver MDH showed higher activity (P<0.05) in birds on malic acid treatments, but IDH activity did not showed significant difference (P>0.05) between treatments. In the second experiment liver MDH activity did not showed any significant difference, but IDH activity was increased (P<0.05) by malic acid consumption. The results of these trials showed that malic acid have the potential for alteration serum metabolites and liver enzyme activities but with different pattern in egg or meat type chickens.

Key words: Chicken, cholesterol, triacylglycerols, MDH, IDH

Introduction

Malic acid takes its name from *Malus*, the Latin for apple, in which it is by far the major organic acid (Jamin, 2000). In the body, peripheral malate derives from food sources and from synthesis in the citric acid cycle. It plays an important role in generating mitochondrial ATP both under aerobic (Cheeseman and Clark, 1988) and hypoxic (Wiesner *et al*., 1988a; Hoehl *et al*., 1987) conditions. Under aerobic conditions, the oxidation of malate to oxaloacetate provides reducing equivalents to the mitochondria by the malate-aspartate redox shuttle (Cheeseman and Clark, 1988). Under anaerobic conditions, with an excess of cytosolic reducing equivalents, inhibition of glycolysis occurs. By its simultaneous reduction to succinate and oxidation to oxaloacetate, malate is capable of removing cytosolic reducing equivalents, thereby reversing inhibition of glycolysis (Hoehl *et al*., 1987; Wiesner *et al*., 1988b). Through the action of malic dehydrogenase followed by transamination reactions, malate is converted to aspartate, and substrates necessary for initiating transmitochondrial exchange of metabolites through the malate-aspartate shuttle are regenerated. In avian species, the liver is the main site for the de novo synthesis of fatty acids (Goodridge and Ball, 1967; Goodridge,1968a; Leveille *et al*., 1968). Most of the NADPH necessary for the synthesis of fatty acids in birds is believed to be derived from the activity of malate dehydrogenase enzyme (Goodridge and Ball, 1966; Goodridge, 1968b; Tanaka *et al*., 1983) because the hepatic monophosphate-shunt dehydrogenases appear not to play an important role in chick hepatic lipogenesis (Goodridge and Ball, 1966; Goodridge, 1968b; Romsos and Leveille, 1974). The activity of hepatic MDH enzyme is highly positively correlated with the rate of fatty acid synthesis, the percentage of body fat and the percentage of abdominal fat in chicks (Tanaka *et al*., 1983; Grisoni *et al*., 1991; Pfaff, 1977; Yeh and Leveille, 1969). Goodridge *et al*. (1989, 1996) demonstrated that nutritionally and hormonally induced changes in MDH enzyme activity were accompanied by comparable changes in enzyme synthesis and in the abundance of MDH enzyme mRNA In addition, IDH-NADP may function as both a residual source for the provision of NADPH and as a source of a coreactant for transamination, (Rosebrough *et al*., 1999). The reasons by which exogenous malic acid may affect on chick hepatic enzyme activity are unclear. Therefore, the current study was conducted to determine the effects of malic acid consumption on chicken hepatic enzyme activity and blood serum triacylglycerols and cholesterol concentration. In this regard knowledge about the role of dietary malic acid on blood serum metabolites is lacking.

Materials and Methods

Animals and treatments: In the first experiment, 1-d-old male egg type chickens were housed in grouped pen and received a corn-based diet. At 2 d of age, twenty chickens were weighed and distributed into four homogenous experimental groups and housed in four pens (five chicks per pen). The pens were 60 × 50 cm. The light was continuous during the experiment. The corn-based diet was formulated according to the nutritional requirements for chickens (NRC, 1994). Diet was fed in mesh form and contained no growth factors, coccidiostats, exogenous enzymes, or antibiotics. Malic acid was added to the water and offered to chicken freely from first to end of experiment with constant concentration in entire experiment. The treatments were zero (as a control), 0.05, 0.10, and 0.15 percent of malic acid which dissolved in water and given to them in waterer pan. Feed and water were supplied ad libitum throughout the entire experiment.

In the second experiment, two hundred and fifty 1-d-old commercial broiler chickens (Ross) were housed in floor pens containing litter composed of wood shaving and received a corn-based starter diet. Four or five days after hatching, the chicks were sorted and those with extreme weights discarded. After sorting, the chicks were randomly assigned to 16 pens each consisting of 12 birds. The room temperature was gradually decreased from 32°C at d1 to 24°C at d 22. The chicks were fed with three type diets consisted starter, grower and finisher. The lighting regimen and malic acid treatments were same as experiment 1.

Collection of samples: All birds in Exp. 1 were killed at 11.00 hours on d21 and two birds (one male and another female which phenotypically selected) from each pen were killed at 06.00 hours on d56 in Exp. 2, by cutting the carotid artery and blood has been taken from these artery. The blood samples were centrifuged for 15 min at 2500 × g, and serum was harvested and stored at -80°C.

Livers were excised rapidly, washed in 155 mM NaCl to remove exterior blood and debris. Total livers in Exp. 1 and portions of liver in Exp. 2 were immediately chilled and homogenized (1, 10 wt/vol) in 100 mM HEPES (pH 7.5) - 3.3 mM β-mercaptoethanol and centrifuged at 14,000 g for 30 min (Rosebrough *et al.*, 1999). The supernatant fractions were kept at -80°C until analyzed for enzyme activity.

Enzyme Assays: The activities of malate, nicotinamide adenine dinucleotide phosphate oxidoreductase-[decarboxylating] (MDH-NADP) and isocitrate, nicotinamide adenine dinucleotide phosphate oxidoreductase [decarboxylating] (IDH-NADP), were estimated. The activity of MDH-NADP was determined by the method of Rosebrough *et al.* (1999). Reactions contained 50 mM HEPES (pH 7.5), 1 mM NADP, 10 mM $MgCl_2$ and the substrate, 2.2 mM L-malate (disodium salt) in a total volume of I mL. Portions (50 µL) of the 14,000 × g supernatants (diluted 1:10) were preincubated in the presence of the first three ingredients. Reactions were initiated by adding the substrate and following the rate of reduction of NADP at 340 nm at 30°C. The activity of IDH-NADP activity was determined by the method of Rosebrough *et al.* (1999). Reactions contained 50 mM HEPES (pH 7.5), 1 mM NADP, 10 mM $MgCl_2$ and the substrate, 4.4 mM DL-isocitrate in a total volume of 1 mL. Portions (50 µL) of the 14,000 × g supernatants (diluted 1:10) were preincubated in the presence of the first three ingredients. Reactions were initiated by adding the substrate and following the rate of reduction of NADP at 340 nm at 30°C. The amount of activity was corrected for the amount of protein in the sample. Enzyme activities are expressed in unit of nanomoles of NADP reduced per minute per mg protein in the extract, under the assay conditions. Protein concentration in the sample was determined using the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Chemical measurements: Total fat contents of liver, was determined by extraction of samples with petroleum ether. The determination of nitrogen in the liver was performed with the macro-Kjeldahl method. The glycogen content in the liver samples was measured as described by Djawdan *et al.* (1998). Serum samples were also analyzed for triacylglycerols using an enzymatic and colorimetric procedure (Kit 10-525, Ziestchem Diagnostic kit, Tehran, Iran) and for cholesterol by an enzymatic procedure (Kit 10-508, Ziestchem Diagnostic kit, Tehran, Iran).

Statistical analysis: The complete randomized model was used to analyze data. The experimental design for Exp.2 was a completely randomized one with a 4 × 2 factorial arrangement of treatments. Each of four treatments was replicated four times per sex (n=4). The data were analyzed using general linear model procedure of SAS (1988). Duncan's multiple range test (SAS, 1988) ($P<0.05$) was used to test the significance of difference between means. A correlation among parameters was determined and correlation coefficients were tested using a t-test (SAS, 1988). Values are given as means SEM, and the homogeneity of variance was checked.

Results and Discussion

Table 1 summarizes the effects of different levels of malic acid on live weight and some serum metabolite of chickens in both experiments. Significant increase ($P<0.05$) of triacylglycerol was observed for chicks on 0.05 percent malic acid. These data showed no

Table 1: Exp. 1 and 2. Influence of malic acid concentration on the final body weight, liver percentage, serum triacylglycerols, and total cholesterol

	Malic acid concentration (%)					Sex		
	0	0.05	0.10	0.15	SEM	Male	Female	P*
Exp. 1								
Body weight[1] (g)	129.5	115.9	122.6	118.6	4.56	-	-	-
Liver (%)	3.31	3.30	3.18	3.03	0.114	-	-	-
Triacylglycerol (mg/dl)	55.8[b]	123.0[a]	75.3[b]	70.1[b]	12.01	-	-	-
Cholesterol (mg/dl)	139.6[c]	186.4[b]	231.1[a]	223.9[a]	12.21	-	-	-
Exp. 2								
Body weight[2] (g)	2600	2530	2525	2470	54.4	2606	2456	0.0110
Liver (%)	1.89	1.95	1.81	1.89	0.080	1.89	1.88	0.8965
Triacylglycerol (mg/dl)	50.3[b]	74.6[a]	50.5[b]	36.4[b]	5.30	50.4	50.5	0.3477
Cholesterol (mg/dl)	85.0[a]	69.4[b]	79.9[ab]	82.8[ab]	4.80	85.7	72.9	0.01341

Final body weight in 21d old male egg type chicken. 2 Final body weight in 56d old broiler chicken. ab Means in row with no common superscript differ significantly (P<0.05). * Probability

Table 2: Exp. 1 and 2. Specific activities of hepatic enzyme for egg and meat types chicken given malic acid in different concentration

	Malic acid concentration (%)					Sex		
	0	0.05	0.10	0.15	SEM	Male	Female	P*
Exp. 1								
MDH-NADP[1]	135.8[b]	154.3[a]	155.5[a]	160.7[a]	5.260	-	-	-
IDH-NADP[2]	150.2	167.5	143.8	166.2	9.224	-	-	-
Exp. 2								
MDH-NADP	130.8	141.7	139.4	130.5	8.37	137.9	133.9	0.5924
IDH-NADP	128.4[b]	153.3[ab]	181.0[a]	127.0[b]	10.16	165.6	129.2	0.0015

[1, 2]Enzyme activity is noted nanomoles NADP reduced/min per mg protein in the extract. [ab] Means in row with no common superscript differ significantly (P<0.05). * Probability.

significant difference (P>0.05) between control and 0.10 and 0.15 percent malic acid for the serum triacylglycerols concentration. The condition for cholesterol is completely different in both experiments. In Exp. 1 use of malic acid showed increase in cholesterol level in blood serum (P<0.05). In contrast, birds on malic acid treatment in Exp. 2 showed lower (P<0.05) or same values for serum cholesterol. In this case significant difference was observed between the sex (P=0.0134). The reason for this partly is related to the type of the chickens (broiler vs egg type) and mainly is related to different age (21d vs 56d). Changes in blood and liver cholesterol contents are more frequently observed, perhaps because blood (serum) and liver cholesterol belong to the "fast turnover cholesterol pool" (Field *et al*., 1960; Chobanian and Hollander, 1962, Konjufca *et al*., 1997; Wagner and Clarkson, 1974) reported that age and sex have a bearing on the cholesterol metabolism and cholesterol concentration in pigeons. The present findings for higher serum cholesterol levels in 21d old male egg type birds in comparison with same age but meat type birds (Konjufca *et al*., 1997) are in agreement with finding (Lorenz *et al*., 1938) that egg type birds have higher blood cholesterol levels than meat type or mature roosters. Similarly, average serum triacylglycerol levels were higher in 21d old egg type birds than in the broiler chickens. Same values has been reported by Qureshi *et al*. (1983) and Konjufca *et al*. (1997).

The incorporation of malic acid in the water significantly affected (Table 2) MDH-NADP activity in Exp. 1 and IDH-NADP activity in Exp.2 (P<0.05). The use of a metabolite of the citric acid cycle to correct an enzymatic deficiency of the respiratory chain has proven successful in the case of NADH coenzyme QI0 oxidoreductase deficiency (Kobayashi *et al*., 1987). Clinical improvement was observed following succinate administration to bypass the deficient enzymes in the respiratory chain. A similar approach could be used to overcome a deficiency of malate dehydrogenase by supplying bioavailable malate in adequate amounts. Malate is the only metabolite of the citric acid cycle, which correlates positively with physical activity. In rats, exercise-induced mitochondrial respiration was associated with increased malate levels only, with the other key metabolites remaining unchanged (Bobyleva-Guarriero and Lardy, 1986).

In rats, the oral administration of potassium malate increases anaerobic endurance, measured by

Table 3: Liver composition in birds on Exp. 2

	Malic acid concentration (%)					Sex		
	0	0.05	0.10	0.15	SEM	Male	Female	P*
Dry matter (%)	29.0[a]	28.6[ab]	28.2[ab]	27.5[b]	0.42	27.9	28.7	0.0936
Glycogen (%)	11.2[ab]	12.5[a]	10.0[ab]	8.4 [b]	1.29	9.9	11.2	0.1645
Crude protein (%)	65.8[b]	65.8[b]	70.3[ab]	73.1[a]	1.68	66.9	70.6	0.0336
Ether extract (%)	17.1	15.4	13.6	12.4	1.61	13.3	15.9	0.1267

[ab]Means in row with no common superscript differ significantly (P<0.05). * Probability.

Table 4: Correlation coefficient between parameters in data from Exp. 1

Item	MDH	IDH	TAG	Chol
MDH	1.0000[a] (0.0000[b])	-0.266 (0.256)	0.102 (0.669)	0.444 (0.050)
IDH		1.0000 (0.0000)	0.073 (0.761)	-0.013 (0.957)
TAG			1.0000 (.0000)	0.001 (0.996)

MDH: liver MDH-NADP activity, IDH: liver IDH-NADP activity, TAG: serum triacylglycerol mg/dl, Chol: serum cholesterol mg/dl.
a Coefficient of correlation. b Level of probability.

swimming time prior to exhaustion, without a concomitant increase in carbohydrate and oxygen utilization (Dunaev *et al*., 1988). This effect of malate showed a dose-response relationship with doubling of swimming time at 250 mg per kg body weight. However, at a higher dosage, a decrease in effectiveness of malate as observed probably due to depletion of other key substances. The above studies suggest that malate has carbohydrate and oxygen-sparing effects.

The effects on IDH-NADP activity did not showed linearity increase due to malic acid concentration increased. In this regard, liver IDH-NADP activity in Exp. 1 and MDH-NADP activity in Exp. 2 did not showed any significant (P>0.05) difference in relation to malic acid concentration in the water consumption. Effect of sex on liver IDH-NADP activity was showed significantly difference (P=0.0015) and interaction of sex in treatment (Fig. 1) was also significant (P=0.0017). The values for MDH-NADP activity was in agreement with finding Balnave (1975) who has worked on laying hens, but the values of the IDH-NADP activity which was reported by him is about two times more than whatever funded in present studies. Reduction in isocitrate dehydrogenase activity, leading to an accumulation of citric acid, which is a positive effector of acetyl Co A carboxylase, key enzyme in fatty acid biosynthesis (Dousset *et al*., 1987). In this regard Rikans *et al*. (1991) has been reported that cytosolic superoxide dismutase and glutathione peroxidase activities in rat liver displayed sex-dependent variations in activity but were unaffected by aging. The present study has been showed that the IDH-NADP activity is partly controlled by sex characteristics.

Liver composition in chickens on Exp. 2 has been shown in Table 3. Higher dry matter and fat content along with lower protein content in the liver was belonged to birds on control treatment (P<0.05). Lowest dry matter and glycogen (P<0.05) and highest protein content in the liver have been shown in birds on 0.15% malate concentration. Effect of sex, only was showed for protein concentration (P=0.0336). Birds have the ability to store large quantities of excess energy (in the form of triglycerides) in liver, adipose tissue and in yolk of developing oocytes (Hermier, 1997). Lipogenesis (i.e., the conversion of glucose to triglycerides) takes place primarily in the liver of birds (Leveille *et al*., 1975) and involves a series of linked, enzyme-catalyzed reactions including glycolysis, the citric acid cycle and fatty acid synthesis. Hepatic lipogenesis is subject to both nutritional and hormonal control and this metabolic process is highly responsive to changes in the diet (Hillgartner *et al*.,1995; Kersten, 2001). Adipose tissue serves primarily as a storage site for lipid with little lipogenesis occurring in this tissue (Hermier, 1997). Differential lipogenic capacity of liver vs. adipose tissue in birds is a function of the expression of a key transcription factor, sterol regulatory element binding protein-1(Richards *et al*., 2003). In another study, Latour *et al*. (1994) has been reported that chicks given 7% added lard had lower liver fat throughout the trial than chicks fed no added lard. Additionally, these chicks had a higher body protein content at 6 and 7 d of age.

The data from the both experiments were pooled separately for each parameter and correlation coefficients with levels of significant probability related to these data are also shown in Table 4 and 5.

In Exp.1 (Table 4) with the exception of the correlation coefficient between MDH with serum cholesterol, no significant correlation was observed throughout the all parameters (P>0.05). However, between MDH and IDH activities a negative correlation was observed, but weak probability (P=0.265) cannot explain the close relationship between two enzymes.

In Exp. 2, ten negative correlation coefficients were found (Table 5). Among them, only the correlation coefficient for liver protein and liver fat, liver glycogen, and serum triacylglycerol along cholesterol and liver glycogen were

Table 5: Correlation coefficient between parameters in data from Exp. 2

Item	MDH	IDH	TAG	Chol	Gly	CP	Fat
MDH	1.0000[a]	0.132	0.243	0.172	-0.114	0.106	-0.036
	(0.0000[b])	(0.471)	(0180)	(0.345)	(0.536)	(0.565)	(0.845)
IDH		1.0000	0.235	0.033	-0.023	0.173	-0.256
		(0.0000)	(0.195)	(0.857)	(0.899)	(0.345)	(0.157)
TAG			1.0000	-0.164	0.646	-0.690	0.296
			(0.0000)	(0.368)	(0.000)	(0.000)	(0.099)
Chol				1.0000	-0.465	0.206	0.140
				(0.0000)	(0.007)	(0.257)	(0.445)
Gly					1.0000	-0.602	-0.076
					(0.0000)	(0.000)	(0.677)
CP						1.0000	-0.743
						(0.0000)	(0.000)

MDH: liver MDH-NADP activity, IDH: liver IDH-NADP activity, TAG: serum triacylglycerol mg/dl, Chol: serum cholesterol mg/dl, GLY: liver glycogen %, CP: liver crude protein %, Fat: liver fat %. a Coefficient of correlation. b Level of probability.

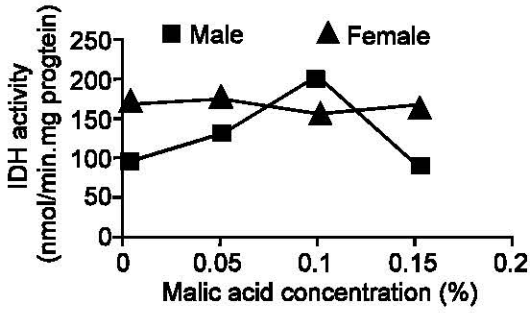

Fig. 1: The interaction between sex and treatment (malic acid concentration) in Exp. 2 (P=0.0017).

significant (P<0.05). A positive significant correlation was observed between liver glycogen and serum triacylglycerol (P<0.05). The negative correlation coefficient between IDH activity and liver fat was observed (P=0.157). This finding reveal that reduction in isocitrate dehydrogenase activity, leading to an accumulation of citric acid, which is a positive effector for fatty acid biosynthesis (Dousset *et al*., 1987).

The coefficient of determination (r^2) showed about 20% cooperative action between MDH activity and serum cholesterol (Exp. 1, P<0.05), but in broiler chicken (Exp. 2) this cooperation was lower than 3% and was not significant (P>0.05). As a mentioned earlier the effect of malic acid treatment on MDH-NADP activity was significant (Exp. 1; P<0.05). On the other hand, the NADPH necessity in several step during the cholesterol biosynthesis is believed to derived from the activity of MDH (Goodridge and Ball, 1966; Goodridge, 1968a; Tanaka, *et al*., 1983) because the hepatic monophosphate-shunt dehydrogenase appear not to play an important role in chick hepatic lipogenesis (Goodridge and Ball, 1966; Goodridge, 1968b; Tanaka, *et al*., 1983).

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