Poultry Science Journal
ISSN: 2345-6604 (Print), 2345-6566 (Online) http://psj.gau.ac.ir
DOI: 10.22069/psj.2021.19005.1685

Effects of Extra Nutritional Vitamin D_3 Levels on Physiological Response of Broiler Chicks Exposed to Repeated Lactic Acidosis

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Poultry Science Journal 2021, 9(2): 197-206

Keywords
Vitamin D_3
Lactic acidosis
Broiler chicken
Sudden death syndrome

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Article history
Received: March 4, 2021
Revised: June 28, 2021
Accepted: August 2, 2021

Abstract

A number of 144 male Ross-308 broiler chicks (18-day old) were used to investigate the effects of extra nutritional vitamin D_3 levels on the physiological response of broiler chicks exposed to lactic acidosis stress. Effects of the six experimental treatment consisting subcutaneous (SC) injection of 0 (control$^-$ and control$^+$), 20000, 30000, 40000, and 50000 IU of vitamin D_3 were examined in four replicates of six birds in a complete randomized block design during days 21 to 29 of age. Lactic acidosis is a form of metabolic acidosis that begins when a bird overproduces or underutilizes lactic acid, and their body is not able to adjust to these changes. Liver fat percentage decreased in the birds receiving 50000 IU of vitamin D_3 compared with the control birds ($P < 0.036$). Administration of 30000 IU vitamin D_3 enhanced serum concentration of creatinine by 18.52 percent compared with the control$^+$ birds ($P < 0.0001$). Injection of 50000 and 40000 IU vitamin D_3 decreased serum concentration of lactate dehydrogenase by 24.99 and 19.81 percent, respectively, compared with the control$^+$ birds ($P < 0.0003$). The mean serum activity of alanine aminotransferase in the birds receiving 20000 IU vitamin D_3 was greater by 28.57, 18.84, and 17.32 percent, compared with the control$^+$ birds and those received 40000 and 50000 IU, respectively ($P < 0.008$). It was concluded that administration of extra nutritional vitamin D_3 up to 50000 IU per bird resulted in broad but inconsistent changes in the blood biochemical and enzymatic parameters in birds exposed to lactic acid. No firm evidence was found to suggest a protective effect vitamin D_3 on the incidence of sudden death syndrome in broilers during lactic acidosis.

Introduction

Lactate is the end product of the anaerobic glycolysis pathway (Chen *et al*., 2017). It is produced in many tissues including red blood cells, skin, adipose tissues, muscles, gastrointestinal tract, and the central nervous system (Wilson, 1956; Jansson *et al*., 1990; Kamel *et al*., 2020).Taking into consideration the several sources of lactate generation in body, lactatemia or lactic acidosis occurs as a result of heavy exercise, exercise at high altitude levels, pulmonary insufficiency, and cardiac failure (Schreurs and Schaafsma, 2010) . Some of these conditions may frequently happen in commercial broilers raised in the crowded condition and inadequate ventilation. In fact, poor management practice could lead to reduced oxygen availability but high CO_2 and NH_4 levels in a broiler house, where chickens use anaerobic glycolysis as a main metabolic pathway of carbohydrates and thus lactic acidosis will inevitably happen (Kerr and Edward, 2019), In addition, in practical poultry farming with commercial fast-growing strains of broilers, the disruption of the balance between the growth of oxygen-consuming organs (such as the breast muscle) and the oxygen-supplying organs (heart and lungs)

Please cite this article as Zeinab Boroumandnia, Heshmatollah Khosravinia, Babak Masouri & Bahman Parizadian Kavan. 2021. Effects of Extra Nutritional Vitamin D_3 Levels on Physiological Response of Broiler Chicks Exposed to Repeated Lactic Acidosis. Poult. Sci. J. 9(2): 197-206.

can lead to hypoxia, thereby developing serious metabolic disorders like ascites (Janice and Balog, 2003). In the human model, obesity and diabetes are two important risk factors for lactic acidosis (Schreurs and Schaafsma, 2010), conditions that can be conceivable for the birds with a greater body mass in a broiler house.

It was shown that the adverse effects of lactic acidosis on broiler chickens exposed to hypoxic conditions might be alleviated by providing the birds with extra nutritional levels of certain nutrients endeavoring cardiopulmonary as well as nervous system metabolism as the two most vulnerable body systems to pH fluctuations (Hulan *et al*., 1980; Whitehead and Randall, 1982; Campbell and Classen, 1989; Cherian, 2007). A growing body of evidence suggests that vitamin D_3 deficiency may be an important factor in creating cardiovascular diseases and immune system failure (Agarwal *et al*., 2011; Camici *et al*., 2013). Vitamin D_3 increases nitric oxide generation through enhanced nitric oxide synthase activity (Dursun *et al*., 2013). Vitamin D_3 also reduces inflammation and biomarkers of oxidative stress (Brandi, 2008) and can prevent the progression of heart failure via modulating blood pressure (Zoccali and Mallamaci, 2014). Additionally, there are studies suggesting vitamin D_3 deficiency has a negative impact oneurodevelopmental change during the fetal period (Feron *et al*., 2005). In vitro experiments also noted that certain antimicrobial responses are vitamin D dependent (Liu *et al*., 2006).

These findings are a part of the existing knowledge supporting our hypothesis that vitamin D_3 can improve heart health and reduce the adverse effects of lactic acidosis. The current-management system in broiler production further support our idea in which fast-growing broiler chickens are often predisposed to vitamin D_3 deficiency since they are raised in intensive indoor flocks with no exposure to sun light and grown on diets with ingredients already depleted from vitamin D_3 due to long duration of transportation, inventory storage, and feed processing methods. Therefore, this study aimed to investigate the effects of parenteral administration of vitamin D_3 on adaptive physiological responses to lactic acidosis in broiler chickens.

Materials and methods

Birds and diets

All procedures carried out in this experiment were approved by the Animal Care and Use Committee of Lorestan University, Khorramabad, Iran. One hundred forty four 18-day-old Ross-308 male broiler chicks were used in this study. The birds were chosen from a flock raised in power ventilated grow-out house where they were fed on a pre-starter (1 to 10 days) and a starter (11 to 20 days) pelleted diet (Table 1). The initial brooding temperature was held at 32 °C for the first three days and then gradually lowered to 25 °C by the end of the experiment. The chicks had free access to feed and water throughout the raising period. Photoperiods were maintained at 24 h during the first day and decreased to 23 h a day for the remainder of the trial. On day 18, experimental birds were chosen and distributed in 24 pens to evaluate the effects of 6 treatments with 4 replicates of 6 birds each in a randomized complete block design. Chicks spent three days for acclimatization and continued starter diet consumption. On day 21, feeding of the grower diet (Table 1) began and birds were subjected to the 6 experimental treatments including; a negative control (sham injection), a positive control (injection of 0.5 mL of olive oil) and subcutaneous (SC) injection of 20,000, 30,000, 40,000, and 50,000 IU of vitamin D_3 (Caspian Tamin Co. Iran) in 0.5 mL of olive oil into the neck of the birds. For induction of lactic acidosis, all treated birds received 0.2, 0.2, and 0.3 mL of intervenous lactic acid (40%; 100366, Merck Co. Germany) with a period of 48 h between each injection. The administrated lactic acid doses were equivalent to 191, 191, and 286 µL/kg of body weight, respectively. The selected doses were based on a preliminary study (Table 2) and our previous work (Boroumandnia *et al*., 2021). In the preliminary test, lactic acid solution (40%) was injected *i.v.* into the birds in increasing doses from 0.1 to 0.6 mL. Each dose was injected into five chicks with a bodyweight close to the experimental chicks through the wing vein. The chicks were monitored for SDS-like death during 24 h post-injection. All the treated birds were individually weighed and then slaughtered 48 h after the last injection.

Growth measurement

Individual bodyweight and pen-wise feed intake were recorded on days 21 and 29, and average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) were calculated. Mortality was recorded upon occurrence.

Carcass characteristics

On day 29, all the birds were killed by puncturing jugular vein and carotid arteries, then scalded, de-feathered mechanically, and eviscerated manually. The relative weight of internal organs including the heart, liver, lungs, pancreas, spleen, bursa of Fabricius, and thymus were calculated by dividing their weight by the live body weight.

Table 1. Ingredients and nutrient composition of diets.

Ingredients (%)	Pre -Starter (1–10 day)	Starter (11–20 day)	Grower (21–29 day)
Yellow maize	55.77	60.97	64.97
Soybean meal	39.00	34.00	30.00
Oyster shell	1.20	1.10	1.10
Calcium phosphate	1.32	1.09	0.94
Vegetable oil	0.80	1.00	1.20
Salt	0.13	0.13	0.13
Vitamin mix[1]	0.50	0.50	0.50
Mineral mix[2]	0.50	0.50	0.50
Sodium bicarbonate	0.10	0.10	0.10
DL-Methionine	0.21	0.19	0.17
L-Lysine HCl	0.18	0.16	0.15
Methionine +Cystine	0.22	0.20	0.18
L-Threonine	0.07	0.06	0.06
Nutrient composition			
Metabolizable energy (Kcal/kg)	2910	2975	3030
Crude protein (%)	22.45	20.60	19.15
Crude fat (%)	3.54	4.09	4.20
Crude fiber (%)	2.78	2.59	2.56
Calcium (%)	0.96	0.87	0.78
Available Phosphorus (%)	0.48	0.44	0.39
Sodium (%)	0.19	0.19	0.18
Potassium (%)	0.70	0.65	0.65
Chlorine (%)	0.19	0.19	0.19
Methionine (%)	0.56	0.51	0.47
Methionine +Cystine (%)	1.08	0.99	0.90
Threonine (%)	1.44	1.29	1.15
Lysine (%)	0.97	0.88	0.78
Vitamin D_3 (IU)	4800	4320	4000

[1]Vitamin A, 440,000 international units; Vitamin D_3, 160,000 international units; Vitamin E, 1,500 international units; Vitamin K3, 128 mg; Vitamin B1, 74 mg; Vitamin B2, 260 mg; Vitamin B3, 490 mg; Vitamin B5, 1,600 mg; Vitamin B6, 120mg; Vitamin B9, 60mg; Vitamin B12, 0.6mg; Vitamin H2, 4mg; Anti-oxidant, 250mg; Choline chloride, 20,000mg.
[2]Manganese, 4,800 mg; Zinc, 4,400 mg; Copper, 650 mg; Selenium, 12 mg; Iodine, 48 mg; Iron, 2,000 mg.

Table 2. The results from a preliminary study to adjust the lactic acid dose for induction of lactic acidosis in broilers.

Age (d)	Administration route	Criterion[1]	Lactic acid levels (mL)											Lactic acid solution
			0	0.1	0.2	0.3	0.4	0.5	0.6	4	5	6	7	
40	Oral gavaging	SDS-like death	0	-	-	-	-	-	-	0	0	0	0	20%
40	Oral gavaging	SDS-like death	0	-	-	-	-	-	-	0	0	0	0	40%
22	Intravenous injection	SDS-like death	0	0	1	5	5	5	5	-	-	-	-	40%
33	Intravenous injection	SDS-like death	0	0	0	1	5	5	5	-	-	-	-	40%
40	Intravenous injection	SDS-like death	0	0	0	1	3	5	5	-	-	-	-	40%

[1]SDS-like death characterized by Newberry *et al.* (1987).

Liver measurements

Livers from all the slaughtered birds were weighed and subjected to a visual appraisal and scored in a 5-point scale for their appearance color. Normal color was given score one, whereas score 5 was assigned to the most yellowish discoloration (Choi *et al.*, 2012). Livers from all the birds were again appraised based on morphological changes such as external hemorrhage. On a 6-point scale, scores were assigned to each category as follows; livers with normal morphological appearance were given 0, 1 to 10 for sub-capsular petechial hemorrhages, 2 for more than 10 sub-capsular petechial hemorrhages, and 3 to 5 for large and massive hemorrhages (Diaz *et al.*, 1999; Rozenboim *et al.*, 2016). After scoring, each liver sample was used for fat extraction using the method of Folch *et al.* (1957). Briefly, about 1 g of liver tissue was weighed, added to chloroform/methanol (2/1, v/v) mixture in a final volume 20 times the volume of the tissue sample, vortexed for 1 min, and allowed to stand with agitation for 2 h. The separated liquid was filtered through Whatman No. 1 filter paper into a 100 mL 54 graduated cylinder, and 5 mL of 7.5% Potassium chloride solution was added and mixed. After phase separation, the top layer was fully drained off. Total lipids were measured gravimetrically after evaporating the solvent. The samples were then dried and weighed, and the total lipid weight was expressed as a percentage of liver fat relative to the total liver weight. The liver dry matter percentage was assayed gravimetrically by oven drying at 105 °C to a constant mass (AOAC 2005,

930.15). Total ash percentage was tested by combustion of each sample at 550 °C for 8 h (AOAC 2005, 923.03).

Heart measurements

The thickness of the left ventricular wall (LVWT), right ventricle wall (RVWT), and interventricular septum (IVST) were determined at five different locations by a digital caliper (HB-101-111, Guanglu, China) and averaged (Harash *et al*., 2019). Fat, ash, and dry matter percentages for heart tissue were measured as described for the liver.

Blood biochemistry

On day 29 of the experimentation period, a 10 mL blood sample was collected from each bird and kept at 25 ºC for 1 h and then transferred on slush-ice pending serum extraction. Samples of whole blood were centrifuged at 1,800 g for 15 min and collected sera stored at –20 °C pending chemical analysis. The serum samples were were used to assess the activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) enzymes. Concentrations of serum biochemical constituents including glucose (GLU), triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), total protein (TP), albumin (ALB), uric acid (UA), urea, creatinine, lactate, and certain electrolytes including Ca, Cl, and Mg were also determined using diagnostic kits (Pars Azmun, Iran) and with an autoanalyzer (Selects E Autoanalyzer, Sr. No. 8-7140, Vital Pharma BV, Maarheeze, The Netherlands). The autoanalyzer based on enzymatic procedures using diagnostic kits. Concentrations of serum very low-density lipoprotein cholesterol (VLDL-c) was calulated based on the method given by Friedewald *et al*. (1972) (TG/ 5). Serum sodium and potassium concentrations were measured using a flame photometer (480, Corning, USA).

Total antioxidant capacity (TAC) was determined using Randox kit (Randox Laboratories Ltd, UK), and Ransod spectrophotometric kits (Ransod, Randox Laboratories Ltd. UK) were used to assess blood superoxide dismutase (SOD) and glutathione peroxidase (GPX) activities. Lipid peroxidation was assayed by the content of Thiobarbituric Acid Reactive Substances (TBARS) in the serum with malondialdehyde (MDA) assay kit (Kiazist Co. Hamadan, Iran). Nitric oxide (NO) concentration in serum samples was determined by a commercially available NO kit (Novin Navand Salamat Pishtaz Co. Urmia. Iran).

White blood cell differential count

Differential leukocyte counts including lymphocytes (L), monocytes, eosinophils, basophils, and heterophiles (H) were conducted using peripheral blood smears stained with Wright-Giemsa staining using a light microscope (Leica Galeni III. USA) equipped with a camera. The H to L ratio was calculated by the method of Grass and Siegel (1983). Smears were examined to obtain counts of L and granulocytes per 100 leukocytes (Krams *et al*., 2012).

Statistical analysis

The collected data were subjected to analysis of variance using PROC Mixed in Statistical Analysis System, version 9.1 (SAS Institute, 2003). The Tukey test was used for multiple treatment comparisons (Kramer, 1956). Liver scores were subjected to frequency analysis using PROC FREQ in the same statistical analysis software (SAS Institute, 2003). Data on mortality were subjected to the Kruskal-Wallis test using PROC NONPARAMETRIC due to lack of normality (SAS Institute, 2003). The maximum likelihood for type-I error was set at 5% for all tests ($P < 0.05$). Specific orthogonal contrasts (linear and quadratic) were applied to determine the effects of different levels of vitamin D_3.

Results

The results of the preliminary experiment intended to adjust the lactic acid dose for induction of lactic acidosis showed that oral gavage of lactic acid solution (20% and 40%) was not able to induce acute lactic acidosis in the birds on day 40 of age. However, intravenous injection of lactic acid at 0.3, 0.4, 0.5, and 0.6 mL per bird caused acute lactic acidosis indicted by SDS-like death in all birds on days 22, 33, and 40 of age, respectively (Table 2). Administration of vitamin D_3 beyond nutritional requirements through SC injection had no influence on ADG, ADFI, FCR, and mortality in the experimental birds during days 21 to 29 of age (Table 3).

No change in the relative weight of bursa of Fabricius, spleen, thymus, pancreas, lungs, liver, and heart was observed in the broiler chicks treated with different levels of vitamin D_3 compared with the control birds on day 29 of age (Table 4).

Subcutaneous injection of grading extra nutritional vitamin D_3 levels modified liver fat percentage in a quadratic trend ($P < 0.013$). The shame-injected birds and those receiving 50,000 IU vitamin D_3 exhibited greater and lesser liver fat percentages than all other birds, respectively ($P < 0.036$; Table 4). The mean liver dry matter and ash in the birds receiving different levels of vitamin D_3 did not vary compared with the control birds at day 29 (Table 4). No change in mean heart dry matter and ash percentage as well as LVWT, RVWT, IVST was observed in the birds receiving incremental levels of vitamin D_3 compared with the control birds on day 29

(Table 4). The birds receiving 30,000 and 40,000 IU vitamin D_3 had lesser and greater heart fat percentage compared with other birds, respectively ($P < 0.005$; Table 4).

Table 3. Means of average daily gain (g), average daily feed intake (g), feed conversion ratio (g: g) and mortality (%) in broiler chicks exposed to lactic acidosis and received different levels of vitamin D_3 whithin 21 to 29 days.

Treatments	Growth parameters			Mortality[1] (no./total)
	ADG	ADFI	FCR	
0 Control$^-$ (sham injection)	85.08	184.20	2.17	8.33 (2/24)
0 Control$^+$ (0.5 mL of olive oil)	87.13	184.51	2.13	8.33 (2/24)
20000 IU Vit. D_3	81.25	174.92	2.16	20.83 (5/24)
30000 IU Vit. D_3	87.01	182.96	2.12	12.50 (3/24)
40000 IU Vit. D_3	87.75	182.30	2.08	16.67 (4/24)
50000 IU Vit. D_3	87.60	184.62	2.11	8.33 (2/24)
SEM	3.592	6.397	0.102	6.365
P-value				
Vit. D_3	0.782	0.655	0.969	0.629
Linear	0.651	0.795	0.721	0.845
Quadratic	0.509	0.520	0.971	0.258

ADG: average daily gain; ADFI: average daily feed intake; FCR: feed conversion ratio; SEM: standard error of mean.
[1]Data were tested using the nonparametric Kruskal-Wallis test.

Table 4. Means of relative internal organs weight (% of live body weight), liver and heart fat, dry matter, ash percent and cardiovascular parameters (mm) in broiler chicks exposed to lactic acidosis and received different levels of vitamin D_3 whithin 21 to 29 days

Indices	Vit. D_3 (IU)						*SEM*	*P*-value		
	Control$^-$	Control$^+$	20000	30000	40000	50000		Vit. D_3	Linear	Quadratic
Bursa of Fabricius (%)	0.15	0.15	0.16	0.16	0.17	0.15	0.011	0.909	0.649	0.407
Spleen (%)	0.16	0.15	0.16	0.14	0.17	0.15	0.009	0.231	0.254	0.738
Thymus (%)	0.04	0.05	0.05	0.04	0.04	0.04	0.002	0.089	0.069	0.157
Pancreas (%)	0.28	0.26	0.28	0.24	0.26	0.25	0.012	0.070	0.276	0.747
Lung (%)	0.51	0.51	0.53	0.50	0.48	0.52	0.016	0.549	0.652	0.458
Liver (%)	3.07	2.98	3.16	2.98	2.91	2.99	0.102	0.459	0.349	0.822
Fat (%)	8.14^a	7.27^{ab}	7.63^{ab}	7.88^a	7.61^{ab}	6.37^b	0.416	0.036	0.146	0.013
DM (%)	28.21	27.46	27.83	27.91	28.29	27.84	0.346	0.568	0.244	0.292
Ash (%)	4.08	4.41	4.62	4.37	4.58	4.73	0.202	0.203	0.307	0.627
Heart (%)	0.50	0.51	0.51	0.51	0.49	0.52	0.015	0.756	0.989	0.442
LVWT (mm)	5.61	5.75	5.99	5.66	6.08	6.04	0.244	0.511	0.177	0.905
RVWT (mm)	1.84	1.92	1.97	2.10	1.95	2.00	0.102	0.532	0.315	0.988
IVST (mm)	4.91	5.08	5.19	4.98	5.14	4.99	0.191	0.855	0.187	0.327
Fat (%)	5.47^b	5.76^{ab}	6.49^a	5.14^b	6.54^a	5.80^{ab}	0.280	0.005	0.897	0.856
DM (%)	23.50	23.17	24.18	23.75	23.38	23.72	0.375	0.482	0.797	0.321
Ash (%)	4.61	4.69	4.61	4.49	4.70	4.51	0.111	0.689	0.438	0.767

SEM: standard error of mean; DM: dry matter; LVWT: left ventricular wall thickness; RVWT: right ventricular wall thickness; IVST: interventricular septum thickness. Lowercase letters to the right of values within a row denote treatment differences ($P < 0.05$).

Serum concentrations of TG, TC, LDL-c, HDL-c, VLDL-c, TP, ALB, GLU, UA, urea, lactate and NO were not significantly different in the birds receiving extra nutritional vitamin D_3 levels through SC injection on day 29 of age compared with the control birds ($P > 0.05$; Table 5). Administration of 30,000 IU vitamin D_3 enhanced serum concentration of creatinine by 18.52 percent than the control$^+$ birds. Serum concentration of creatinine in the birds receiving 40,000 and 50,000 IU vitamin D_3 lowered by 42.62 and 18.85 percent compared whit the control$^-$ birds, respectively ($P < 0.0001$; Table 5).

Vitamin D_3 exhibited no effect on serum concentration of Na, K, Mg, and Ca compared with the control birds on day 29 of age. Parenteral administration of 20,000 IU vitamin D_3 decreased serum concentration of Cl^- by 5.56 percent compared with the control$^+$ birds ($P < 0.036$; Table 5). No difference was observed in the mean serum activity of GPX, SOD, TAC and MDA of birds receiving extra nutritional vitamin D_3 levels compared with the control birds on day 29 of age ($P > 0.05$; Table 5). Administration of vitamin D_3 had no significant effect on the serum activity of ALP and AST compared with the control birds on day 29. However, SC injection of 50,000 and 40,000 IU vitamin D_3 decreased serum concentration of LDH by 24.99 and 19.81 percent, respectively, compared with the

control birds ($P < 0.0003$; Table 5). Vitamin D_3 injection resulted in a decreased activity of LDH in a linear pattern ($P < 0.001$). The mean serum activity of ALT in the birds receiving 20,000 IU vitamin D_3 was greater by 28.57, 18.84 and 17.32 percent, compared with the control$^+$ birds and those received 40,000 and 50,000 IU, respectively ($P < 0.008$; Table 5). Administration of different extra nutritional vitamin D_3 changed serum ALT activity of the birds in a nonlinear fashion ($P < 0.015$).

Table 5. Means of blood biochemical parameters, electrolytes, antioxidant indices concentration and hepatic enzymes activity in broiler chicks exposed to lactic acidosis and received different levels of vitamin D_3 whithin 21 to 29 days.

Indices	Vit. D_3 (IU)						*SEM*	*P*-value		
	Control$^-$	Control$^+$	20000	30000	40000	50000		Vit. D_3	Linear	Quadratic
TG (mg/dL)	46.28	45.17	47.07	45.00	43.84	43.78	2.713	0.951	0.486	0.766
TC (mg/dL)	113.67	123.56	122.47	125.78	125.00	133.33	4.963	0.151	0.165	0.431
LDL (mg/dL)	53.23	55.99	55.11	59.80	59.11	62.14	3.177	0.391	0.118	0.837
HDL (mg/dL)	56.40	58.95	53.88	53.70	55.70	57.50	2.948	0.794	0.911	0.164
VLDL (mg/dL)	9.26	9.03	9.41	9.00	8.77	8.76	0.542	0.952	0.486	0.766
TP (g/dL)	4.24	4.08	4.38	4.03	4.22	4.13	0.154	0.583	0.928	0.977
ALB (g/dL)	1.60	1.59	1.60	1.50	1.57	1.58	0.062	0.815	0.891	0.382
GLU (mg/dL)	136.08	143.30	115.33	136.59	137.89	148.08	8.039	0.110	0.161	0.040
UA (mg/dL)	8.19	6.99	6.84	8.23	7.21	7.72	0.411	0.069	0.162	0.476
Urea (mg/dL)	5.17	5.06	5.29	4.89	4.37	4.07	0.324	0.109	0.486	0.766
Creatinine(mg/dL)	0.122^{ab}	0.110^{bc}	0.115^{abc}	0.135^{a}	0.070^{d}	0.099^{c}	0.007	<0.0001	0.777	0.591
Lactate (mmol/L)	10.38	9.39	9.68	9.73	9.73	9.58	0.395	0.600	0.747	0.558
NO (µmol/L)	8.35	8.63	7.88	8.34	8.41	8.04	0.456	0.886	0.620	0.820
Na (mg/dL)	175.16	154.94	151.83	159.63	157.04	161.17	7.150	0.347	0.439	0.881
K (mg/dL)	8.70	7.94	8.01	7.16	8.10	7.27	0.650	0.597	0.529	0.996
Mg (mg/dL)	2.00	1.83	1.81	1.95	1.80	1.75	0.097	0.410	0.564	0.340
Ca (mg/dL)	7.94	7.14	7.74	7.51	8.10	7.77	0.285	0.189	0.081	0.342
Cl (meq/L)	105.50^{ab}	108.29^{a}	102.27^{b}	107.39^{a}	106.79^{a}	107.67^{a}	1.325	0.036	0.433	0.102
GPX (u/g Hb)	171.10	147.86	175.45	135.20	159.75	169.18	11.807	0.091	0.426	0.474
SOD (u/g Hb)	983.29	990.00	1007.40	972.35	946.98	907.70	65.466	0.846	0.289	0.549
TAC (mmol/L)	1.46	1.30	1.41	1.49	1.41	1.59	0.111	0.479	0.211	0.950
MDA (nmol/mL)	1.66	1.62	1.84	1.54	1.57	1.56	0.095	0.310	0.217	0.728
LDH (u/L)	2593.60^{a}	2329.30^{ab}	2317.90^{ab}	1976.50^{bc}	1867.80^{c}	1747.10^{c}	151.293	0.0003	0.001	0.980
ALP (u/L)	3093.56	2717.93	3280.33	2548.00	2593.88	2801.12	288.537	0.414	0.547	0.947
AST (u/L)	211.72	211.62	218.29	230.49	196.62	216.05	10.857	0.243	0.677	0.595
ALT (u/L)	3.12^{ab}	2.35^{c}	3.29^{a}	2.88^{abc}	2.67^{bc}	2.72^{bc}	0.179	0.008	0.822	0.015

SEM: standard error of mean; TG: total triglycerides; TC: total cholesterol; LDL: low density lipoproteins; HDL: high density lipoproteins; VLDL: very low density lipoprotein; TP: total protein; ALB: albumin; GLU: glucose; UA: uric acid; NO: Nitric oxide; Na: sodium; K: potassium; Mg: magnesium; Ca: calcium; Cl: chlorine; GPX: glutathione peroxidase; SOD: superoxide dismutase; TAC: Total antioxidant capacity; MDA: malondialdehyde; LDH: lactate dehydrogenase; ALP: alkaline phosphatase; AST: aspartate aminotransferase; ALT: alanine aminotransferase. Lowercase letters to the right of values within a row denote treatment differences ($P < 0.05$).

No change in the differential count of monocyte, eosinophil, and basophil percentage was found in the birds receiving vitamin D_3 in a range of 20,000 to 50,000 IU on day 29. Heterophile (H) percentage decreased by 27.74, 23.95 and 27.99 percent in the birds treated with 20,000, 30,000, and 40,000 IU vitamin D_3, respectively, compared with the control birds ($P < 0.008$; Table 6). The birds receiving 30,000 and 40,000 IU vitamin D_3 had greater lymphocyte (L) percentage than control$^-$ birds ($P < 0.039$). The birds receiving 50,000 IU vitamin D_3 also showed a greater H/L ratio than those receiving 20,000 and 40,000 IU vitamin D_3 ($P < 0.028$; Table 6). Administration of vitamin D_3 brought about a nonlinear pattern of change in H and L count as well as H/L ratio ($P<0.001$, $P < 0.005$ and $P < 0.003$, respectively).

The frequency of liver health score 2 (3.57%) and 3 (8.51%) was lower in the birds receiving 20,000 IU of vitamin D_3 compared with the control birds at the age of 29 d. The birds receiving 30,000 IU vitamin D_3 showed a greater frequency for liver health score 2 compared with the control birds ($P < 0.001$; Table 7). The frequency of liver health score 4 was greater (26.32 percent) in the birds receiving 20,000 IU of vitamin D_3 compared with the control birds on day 29. The frequency of liver color score 3, 4 and 5 were influenced by the experimental treatments at the age 29 d. The frequency of liver color score 3 was greater (30.43 percent) in the control$^+$ birds, and it was lesser (4.35 percent) in those received 20,000 IU of vitamin D_3. The birds receiving 20,000 IU vitamin D_3 showed lesser (11.54 percent) frequency for score 4 ($P < 0.001$; Table 7). A lesser frequency for liver color score 5 was observed in the birds receiving 40,000 IU

of vitamin D_3 while a greater frequency for the same score was found in control$^-$ birds and those receiving 20,000 and 50,000 IU of vitamin D_3 ($P < 0.001$; Table 7).

Table 6. Means of heterophile, lymphocyte, monocyte, eosinophil, basophil count and heterophile to lymphocyte ratio (%) in broiler chicks exposed to lactic acidosis and received different levels of vitamin D_3 whithin 21 to 29 days.

Treatments	H	L	M	E	B	H/L
0 Control$^-$ (sham injection)	37.96^{ab}	50.67^{b}	7.18	2.72	1.37	0.82^{ab}
0 Control$^+$ (0.5 mL of olive oil)	40.66^{a}	51.59^{ab}	5.48	2.38	1.31	0.84^{ab}
20000 IU Vit. D_3	29.38^{bc}	57.92^{ab}	8.25	3.14	1.78	0.57^{b}
30000 IU Vit. D_3	30.92^{bc}	59.53^{a}	6.23	3.06	1.22	0.61^{ab}
40000 IU Vit. D_3	29.28^{c}	59.86^{a}	7.37	3.38	1.41	0.53^{b}
50000 IU Vit. D_3	37.21^{abc}	52.11^{ab}	7.75	4.20	1.27	0.89^{a}
SEM	2.934	2.782	1.04	0.56	0.20	0.10
P-value						
Vit. D_3	0.008	0.039	0.224	0.275	0.459	0.028
Linear	0.424	0.729	0.257	0.028	0.475	0.784
Quadratic	0.001	0.005	0.760	0.805	0.527	0.003

H: heterophile; L: lymphocyte; M: monocyte; E: eosinophil; B: basophil; SEM: standard error of mean. Lowercase letters to the right of values within a column denote treatment differences ($P < 0.05$).

Table 7. Frequency of apparent liver health and liver color scores in broiler chicks exposed to repeated lactic acidosis and received different levels of vitamin D_3 whithin 21 to 29 days.

Treatments	Liver health score[1]						Liver color score[2]				
	0	1	2	3	4	5	1	2	3	4	5
0 Control$^-$ (sham injection)	0.00	25.00	10.71	27.66	13.16	0.00	0.00	33.33	21.74	15.38	20.51
0 Control$^+$ (0.5 mL of olive oil)	0.00	50.00	21.43	17.02	15.79	0.00	0.00	33.33	30.43	15.38	15.38
20000 IU Vit. D_3	0.00	0.00	3.57	8.51	26.32	0.00	0.00	0.00	4.35	11.54	20.51
30000 IU Vit. D_3	0.00	25.00	32.14	10.64	13.16	0.00	0.00	0.00	21.74	19.23	12.82
40000 IU Vit. D_3	0.00	0.00	17.86	10.64	15.79	0.00	0.00	0.00	8.70	19.23	10.26
50000 IU Vit. D_3	0.00	0.00	14.29	25.53	15.79	0.00	0.00	33.33	13.04	19.23	20.51
P-value											
Vit. D_3	-	0.09	<0.001	<0.001	<0.001	-	-	0.19	<0.001	<0.001	<0.001
χ^2	-	8.00	140.00	235.00	190.00	-	-	6.00	115.00	260.00	195.00

[1]Liver health score: 0 indicating normal liver, 1 up to 10 sub capsular petechial hemorrhages, 2 for more than 10 sub capsular petechial hemorrhages, and 3 to 5 for large and massive hemorrhages.
[2]Liver color score: 1 indicating normal liver, score from 2 to 5 from dark red to light yellowish red.

Discussion

Vitamin D_3 receptor exists in most tissues such as vascular endothelium, vascular smooth muscle cells, and myocardium (Baeke *et al*., 2010); therefore, this fat-soluble vitamin plays multiple roles in several metabolic pathways. In addition, many extra kidney tissues such as vascular endothelial cells, vascular smooth muscle cells, immune cells, brain and digestive system are able to express alpha hydroxylase enzyme and hence to convert $25(OH)D_3$ to $1,25(OH)_2D_3$ (Zehnder *et al*., 2002). A growing body of evidence suggests that vitamin D_3 deficiency may result in many metabolic impairments including cardiovascular diseases (Agarwal *et al*., 2011; Camici *et al*., 2013).

In the current study with a preliminary trial we were not able to induce acute lactic acidosis (indicating by SDS-like death) in broiler chicken using oral gavaging of 4 to 7 mL of lactic acid solution (20 and 40%). However, we succeeded to create acute lactic acidosis via IV injection of lactic acid in the same birds, indicated by paroxysmal strong muscular contractions, violent flapping and ultimately overturning in all the birds receiving lactic acid beyond 191 µL/kg body weight. The same results have been reported by many previous (Summer *et al*., 1987; Jacob *et al*., 1990; Imaeda, 2000) as well as recently conducted researches mainly in human model (Gillies *et al*., 2019; Radelfahr and Klopstock, 2019; Kamel *et al*., 2020). In the above-mentioned preliminary study, we injected incremental doses of lactic acid into five birds each. The minimum dose causing SDS-like death in the first bird was used in the current study to induce lactic acidosis. With the doses lesser than this threshold dose, birds did not die but showed increased frequency of fatigue, napping behavior and lethargy with a dropped neck. It was shown that the metabolic acidosis created by lactic acid injection inactivated glycolytic enzymes such as phosphofructokinase (PFK), impaired muscle contraction, and created the burning sensation in

working muscles (Kerr and Edward, 2019). We confirm these results evidenced by the increased frequency of napping behavior, failure of balance, strong muscular contractions, ultimately violent flapping and overturning in the low-dose lactic acid treated birds.

Previous works confirmed that young broiler chicks have a tolerance for excess vitamin D_3 as high as 50,000 IU per kg with no adverse effects on performance and bone mineralization (Baker *et al*., 1998). We interested to examine the effects of increasing levels of vitamin D_3, up to such a maximum threshold level on response of the young chicken to repeated lactic acidosis. With respect to productive performance, no adverse effect of extra nutritional vitamin D_3 levels was found on ADG, ADFI and FCR in days 21 to 29 of age, which confirm the previous reports announcing appreciate tolerance in broiler chicken for excess vitamin D_3 as high as 50,000 IU per kg (Baker *et al*., 1998).

Outcomes of the present study for blood biochemistry, enzymes activity and liver variables which have frequently been reported as indicators of stress response in chicken (Puvadolpirod and Thaxton, 2000a,b; Post *et al*., 2003; Khosravinia and Manafi, 2016) revealed that extra nutritional vitamin D_3 levels may support bird's metabolism to overcome acute lactic acidosis stress. However, the results are inconsistent. Serum GLU, CHO, HDL-c, TG, UA, ALB, TP concentrations and greater liver weight as the elicited adaptive responses to stressors in broiler chickens (Puvadolpirod and Thaxton, 2000a) were not differ in the control and the vitamin D_3 injected birds. Vitamin D_3 injection could not modify the pattern of changes occurred in these variables and did not support certain degrees of stress relief evidenced by decreased GLU, CHO, and TG in the treated birds. Our results showed that surplus vitamin D_3 may not modulate or prevent resistant hypertension evidenced by no increase in serum NO levels. Findings of our study, are inconsistent with previous studies in which vitamin D_3 increased NO generation through enhanced nitric oxide synthase activity (Dursun *et al*., 2013; Zoccali and Mallamaci, 2014). This outcome also disagrees results from those studies which show that human cases with higher levels of 2-hydroxy vitamin D_3 (measurable blood vitamin D_3) have lower blood pressure, glucose and lipid levels (Deleskog and Ostenson, 2015; Juraschek *et al*., 2015).

It is rational to suggest that poor oxygen supply due to deficient growth of certain internal organs (heart, lung, etc.) coincide with increasing amassing of CO_2 and NH_4 resulting from improper ventilation in the broiler house may result in a persistent hypoxia and declined aerobic metabolism in the birds. These reactions result in the production of lactate from pyruvate by LDH and increased lactic acid production leads to change in blood pH, acidosis and finally cardiovascular system damage (Meshram and Bijoy, 2017). The same situation may worsen when birds have free access to feed since more lactic acid is likely produced from digestion and microbial fermentation processes in the gastrointestinal tract. Such lactic acid, in turn, may diffuse into blood stream and influence the acid-base balance and SDS-like death incidence. These conditions interpret the more prevalence of SDS in the birds with greater body weight, in males than females or in the well-nourished flocks.

Based on the findings of the present study, it can be concluded that intravenous injection of lactic acid can be used as an efficient experimental method to induce lactic acidosis stress in broiler chicken. Administration of vitamin D_3 in doses beyond nutritional requirements up to 50,000 IU resulted in extensive but inconsistent changes in blood biochemical and enzymatic parameters in the lactic acidosis-stressed birds. However, our results failed to show extra nutritional vitamin D_3 may reduce the risk of cardiovascular diseases including acute lactic acidosis and SDS-like death in broiler chicken.

Acknowledgments

The authors would like to thank the Research Directorate of Lorestan University for partial financial support.

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