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The effect of *in ovo* feeding of L- methionine on carcass traits, small intestine morphology, and blood metabolites of day-old broiler chicks

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Abstract

Introduction: Methionine, as the first limiting amino acid in poultry, is necessary for protein synthesis and growth. Several studies evaluated the impact of *in ovo* injection of DL- methionine, but there is no report regarding the *in ovo* injection of L- methionine. **Aim:** Therefore, the purpose of this study was to investigate the effect of *in ovo* feeding of L- methionine on weight, carcass traits, small intestine morphology, and blood metabolites of day-old broiler chicks. **Materials and method:** To achieve the aim of the study, 240 broiler breeder eggs (Ross 308) were used based on a completely randomized design with eight experimental treatments and 30 eggs per each treatment. Experimental treatments included: six levels of L- methionine (0.19, 0.38, 0.57, 0.76, 0.95, and 1.14% L-methionine), a sham control (injection of distilled water), and a control (non- injected group), which were injected into the amniotic fluid at 14 days of incubation. After hatching, chicks were weighed, blood samples were collected, and they were humanly euthanized to evaluate carcass traits and small intestine morphology. After that, serum of blood samples was collected and used for measuring metabolite concentrations. **Results:** The results of the study indicated that *in ovo* feeding of L-methionine increased carcass efficiency and thigh, breast, gizzard, and thymus weight ($P<0.05$), but did not influence hatchability ($P>0.05$). In addition, reducing effects of L- methionine treatments on serum glucose, triglyceride, and blood urea nitrogen were observed ($P<0.05$). Moreover, *in ovo* injection of L- methionine improved length and weight of small intestine, and duodenum and jejunum morphology parameters (villous height, villous height/crypt depth ratio, crypt diameter, and crypt depth), ($P <0.05$). **Conclusion:** According to the results of this study, *in ovo* feeding of 0.19% L-methionine had an improving effect on hatchability, carcass traits, blood metabolites, immune system organs, and morphology of small intestine; therefore, 0.19% L- methionine is an advisable level for *in ovo* feeding.

Keywords: Blood metabolite, Broiler chick, *In ovo* feeding, L- methionine, Small intestine

Introduction

During the last stages of incubation, avian mortality mostly happens due to the lack of enough nutrients (especially amino acids), which reduces hatchability, embryonic development, and muscle growth (Ebrahimi et al. 2018a). Also, a delay in providing feed and water after hatching can worsen the adverse effects of poor nutritional status on growth (Ebrahimi et al. 2018b, 2019). Accordingly, a new technique of *in ovo* feeding was developed to overcome these nutrient limitations (Li et al. 2016; Ebrahimi et al. 2017; Gao et al. 2018). It was indicated that *in ovo* feeding improves chicks' muscle growth (by increasing hyperplasia of embryonic myofibers), immunity, small intestine morphology, and gut function (Bhanja et al. 2012; Goel et al. 2016; Ebrahimi et al. 2019; Ebrahimi et al. 2018a; Gao et al. 2018).

Methionine is considered the first limiting amino acid for poultry, which contributes

mainly for protein synthesis (Annongu et al. 2014; Coskun et al. 2014; Shen et al. 2015). Previous studies indicated the impact of methionine on performance and muscle growth (Coskun et al. 2014; Shen et al. 2015; Razani et al. 2017; Sigolo et al. 2019). Methionine also plays several other critical roles in the body, including: 1- Role in synthesis of polyamines (spermidine and spermine) and so, improving growth and protein synthesis (Bouyeh 2012). 2- Role as a methyl donor for cellular methylation reactions required for the synthesis of carnitine, choline, epinephrine, and creatine (Mohammadrezaei et al. 2015). 3- Role in biosynthesis of L-carnitine, which mediates β-oxidation of fatty acids (Bouyeh 2012). 4- Role as a source of sulfur in body, which is necessary for the synthesis of cysteine, taurine, and carnitine (Saki et al. 2014). 5- Role as a precursor of glutathione and protection against oxidative stress (Bouyeh 2012; Elwan et al. 2019). 6- Role in the synthesis of creatine, which increases muscle fiber size and muscle growth (Ahmed and Abbas, 2015). 7- Role in humoral and cellular immunity as well as lymphoid organ growth (Bhanja et al. 2012; Saki et al. 2014). 8- Role in the tissue expression of IGF-I and growth (Elwan et al. 2019).

Only L isomer of all amino acids has a biological function in animal tissues, but methionine is an exception in poultry. Birds can utilize both D and L isomers of methionine because of the presence of converting enzymes in their liver and kidneys (Thwaites and Anderson 2007). Based on the previous studies, gastrointestinal tract can only use L-methionine (Shen et al. 2015). Also, L-methionine is the only isomer of methionine that can be used in muscles for protein synthesis (Shen et al. 2015). In overall, feeding of L- methionine was more effective in improving broiler chickens performance and intestine morphology than DL- methionine (Shen et al. 2015). Furthermore, it was indicated that the expression of the enzymes responsible for converting D- methionine into L- methionine is very low in young birds (Shen et al. 2015). Accordingly, L- methionine may be the only active form of methionine, which can be used by muscles and intestinal cells of chick embryo (Shen et al. 2015). Therefore, *in ovo* feeding of L- methionine may enhance muscle growth and intestinal development. Although several previous studies used *in ovo* injection of DL- methionine and reported higher chick weight and growth performance along with improvement in intestinal development (Coskun et al. 2014, 2018; Saki et al. 2014; Mohammadrezaei et al. 2015; Elwan et al. 2019), there is no report regarding *in ovo* injection of L- methionine on chick characteristics.

Therefore, this study was designed to investigate the impact of *in ovo* feeding of different levels of L- methionine on chick weight, carcass traits, small intestine morphology, and blood metabolites of hatchlings.

Materials and methods

Birds and experimental design

This experiment was confirmed by Animal Care and Use Committee (University of Tabriz, Iran). The experiment was conducted at Veterinary Clinic and Animal Science Labs (University of Tabriz, Tabriz, Iran) and also Histology lab (Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran). Different levels of L- methionine were determined as follows: the level of 0.19% methionine was *in ovo* injected (in a mixture of amino acids) in the experiment of Ohta et al. (2001) and also this particular level of methionine was fed during growth period in the experiment of Shen et al. (2015). Then, this level was considered the first level of this study and other levels were calculated by adding 0.19% to the previous value. Accordingly, experimental treatments included 0.19 (Ohta et al. 2001; Shen et al. 2015), 0.38, 0.57, 0.76, 0.95, and 1.14% L- methionine, control (non- injected group), and sham control (distilled water injected group). For this reason, 240 fertile Ross × Ross 308 broiler breeder eggs were obtained from Khoshkhan broiler breeder farm (Tabriz, Iran) with the age of 32 weeks. Eggs were numbered, weighed, and assigned into 8

experimental groups with the same average weight and placed into the incubator (with the capacity of 360 eggs). During first 18 days of incubation, the incubator was set on 60% humidity and 37.8°C with six rotations per day. During three last days of incubation (d 19-21), eggs were placed into hatchery boxes with individual cells for each egg. Also, the incubator was set on 70% humidity, 37.4°C, and no rotation. *In ovo* injection was performed at the 14 days of the incubation (Bhanja et al. 2012; Salary et al. 2014; Ebrahimi et al. 2017). For this reason, 1.9, 3.8, 5.7, 7.6, 9.5, and 11.4 mg L- methionine (≥98.5% purity; MB0346 Bio-Basic) were dissolved into 1 ml of distilled water. Then, pH was set on 7 and solutions were warmed up to 35°C. After candling, the area around the air cell was lined up by a pencil. Next, the area was disinfected by 70% Ethyl alcohol and a hole was punched (5mm above the air cell) by a special needle. Afterward, 1 ml of solution treatments was injected into the amnion by 24 mm gauge needle. Then, the hole was sealed and eggs were transferred into the incubator. All the procedures were the same for control groups, except that the control received no injection, while the sham control received 1 ml of distilled water. In order to avoid embryo loss through cross contaminations, needle was disinfected after each injection and a different syringe was used for each treatment.

Data collection and measurements

After hatching, chicks in each treatment were individually weighed and hatchability was calculated. Then, blood samples were collected by heart puncture and soon after, chicks were humanly euthanized. Thereafter, samples were centrifuged (3000 rpm for 15 min at 4°C), serum was collected, and samples were stored at -20°C until analyses of metabolites. Next, carcass characteristics were weighed, included: scalped carcass, eviscerated carcass, thigh, breast, proventriculus, pancreas (detached from the duodenal wall), gizzard, heart, liver (without gall bladder), thymus (right lobes), bursa of Fabricius, and spleen. Also, weight and length of duodenum, jejunum, and ileum were measured and total weight and length of intestine were calculated. Afterwards, the relative weight of all mentioned traits to live body weight was calculated.

Serum metabolites (glucose, total protein, urea, cholesterol, and triglyceride) were measured by Enzymatic-Colorimetric method using Sigma Diagnostics reagents (Zist Shimi Co, Iran) and Auto analyzer instrument (Alyson 300, England), (Ebrahimi et al. 2017). Furthermore, blood urea nitrogen (BUN) was computed as follows: BUN= urea/2.14 (Ebrahimi et al. 2017).

Also, tissue samples of duodenum, jejunum, and ileum were stored in 10% neutral buffered formalin for fixation and stored until histological evaluations. Small intestine histological parameters, including: villous height, crypt depth, villous thickness, and crypt diameter were determined using Hematoxylin - Eosin staining method, which has been described in a previous study (Ebrahimi et al. 2017). Finally, villous height to crypt depth ratio was calculated.

Statistical analysis

Hatchability was analyzed based on the LOGISTIC procedure of SAS software (Ver. 9.2). Other data were analyzed by GLM procedure of SAS software with considering covariate effect of egg weight. Also, Tukey-Kramer test was used for comparing treatments. A probability (P) values of ≤ 0.05 were considered statistically significant. Results are expressed as least square means±standard error.

Table 1- The impact of L- methionine *in ovo* feeding on hatchability, chick weight and carcass traits of one day-old broiler chicks

Traits *, 1	Different levels of L- methionine *in ovo* feeding								P-value
	Control	Sham control	0.19%	0.38%	0.57%	0.76%	0.95%	1.14%	
Hatchability (%)	56.67	53.33	63.33	56.67	43.33	56.67	43.33	23.33	0.06
Chick W (g)	44.28±0.75	43.56±0.79	46.73±0.88	45.78±1.01	46.65±1.41	45.91±0.98	46.28±1.63	46.25±1.82	0.25
Chick W to egg W ratio (%)	68.90±1.16	67.87±1.22	72.59±1.36	71.19±1.56	72.48±2.17	71.43±1.52	72.12±2.52	72.09±2.82	0.27
Scalped carcass W (%)	64.87±0.65	64.16±0.59	66.99±0.62	66.43±0.71	66.55±0.99	65.67±0.69	65.97±1.13	64.78±1.27	0.16
Carcass efficiency (%)	28.59±0.54^{b}	29.14±0.49^{ab}	31.29±0.52^{a}	29.93±0.59^{ab}	30.16±0.83^{ab}	31.35±0.57^{a}	30.74±0.94^{ab}	30.63±1.06^{ab}	0.02
Thigh W (%)	9.71±0.16^{c}	9.71±0.14^{c}	10.87±0.15^{a}	10.10±0.17^{bc}	10.28±0.24^{abc}	10.45±0.17^{ab}	9.84±0.27^{bc}	10.04±0.31^{bc}	<0.01
Breast W (%)	2.21±0.08^{c}	2.24±0.07^{bc}	2.71±0.07^{a}	2.75±0.08^{a}	2.67±0.11^{ab}	2.76±0.08^{a}	2.52±0.13^{abc}	2.50±0.15^{abc}	<0.01
Heart W (%)	0.517±0.019	0.538±0.017	0.541±0.017	0.558±0.019	0.568±0.027	0.573±0.019	0.499±0.032	0.508±0.036	0.343
Gizzard W (%)	3.57±0.10^{c}	3.77±0.09^{bc}	3.98±0.09^{abc}	4.12±0.11^{ab}	4.17±0.15^{ab}	4.39±0.10^{a}	3.71±0.17^{bc}	3.59±0.19^{bc}	<0.01
Liver W (%)	1.89±0.06	1.94±0.05	1.93±0.05	1.95±0.06	1.94±0.09	2.06±0.06	1.93±0.09	2.07±0.11	0.53
Proventriculus W (%)	0.566±0.014	0.556±0.012	0.610±0.013	0.584±0.015	0.595±0.021	0.599±0.014	0.567±0.024	0.592±0.026	0.202
Pancreas W (%)	0.069±0.007	0.067±0.006	0.089±0.006	0.089±0.008	0.095±0.011	0.098±0.007	0.094±0.013	0.087±0.013	0.059
Thymus W (%)	0.075±0.007^{b}	0.080±0.006^{b}	0.112±0.006^{a}	0.083±0.007^{b}	0.081±0.009^{b}	0.092±0.007^{ab}	0.098±0.011^{ab}	0.085±0.012^{ab}	0.004
Bursa of Fabricius W (%)	0.062±0.006	0.062±0.005	0.067±0.005	0.058±0.006	0.067±0.008	0.080±0.006	0.083±0.009	0.069±0.010	0.108
Spleen W (%)	0.032±0.004	0.033±0.004	0.033±0.003	0.037±0.004	0.039±0.006	0.032±0.004	0.031±0.006	0.033±0.007	0.957

* Data are included least square means±SE. ^{a,b,c} Different superscripts within the same line means significant differences (P<0.05).

[1] All weight parameters are computed as their relative weight to chick weight for each individual bird. W stands for weight.

Table 2- The impact of L- methionine *in ovo* feeding on blood metabolites of day-old broiler chicks.

Traits *	Different levels of L- methionine *in ovo* feeding								P-value
	Control	Sham control	0.19%	0.38%	0.57%	0.76%	0.95%	1.14%	
Glucose (mg/dl)	214.33±5.78^{a} ^{b}	218.00±5.07^{a}	196.33±7.46^{abc}	187.40±7.46^{bc}	184.73±7.46^{c}	180.00±7.46^{c}	175.33±8.17^{c}	174.50±8.17^{c}	<0.01
Total protein (g/dl)	1.47±0.07	1.55±0.06	1.67±0.08	1.63±0.08	1.66±0.08	1.65±0.08	1.57±0.09	1.65±0.09	0.49
Blood urea nitrogen (mg/dl)	9.49±0.48^{ab}	9.53±0.43^{a}	7.165±0.63^{b}	8.13±0.63^{ab}	8.22±0.63^{ab}	8.13±0.63^{ab}	7.84±0.69^{ab}	8.18±0.69^{ab}	0.04
Triglyceride (mg/dl)	74.40±3.12^{ab}	76.08±2.74^{a}	73.33±4.03^{ab}	73.00±4.03^{ab}	63.36±4.03^{ab}	65.83±4.03^{ab}	59.60±4.42^{b}	72.0±4.42^{ab}	0.03
Cholesterol (mg/dl)	388.50±18.59	384.38±16.31	350.17±24.01	352.83±24.01	370.20±24.01	375.00±24.01	351.80±26.29	374.50±26.29	0.84

* Data are included least square means±SE. ^{a,b,c} Different superscripts within the same line means significant differences (P<0.05).

Table 3- The impact of L- methionine *in ovo* feeding on small intestine weight and length parameters of day-old broiler chicks.

Traits [*, 1]	**Different levels of L- methionine *in ovo* feeding**								P-value
	Control	Sham control	0.19%	0.38%	0.57%	0.76%	0.95%	1.14%	
Duodenum W (%)	2.09±0.11	2.01±0.10	2.15±0.11	2.21±0.12	2.42±0.17	2.44±0.12	2.39±0.22	2.15±0.22	0.18
Duodenum length (cm)	6.63±0.21^{b}	6.68±0.19^{b}	6.79±0.20^{ab}	6.91±0.23^{ab}	7.38±0.32^{ab}	7.63±0.22^{a}	6.95±0.41^{ab}	6.78±0.41^{ab}	0.04
Jejunum W (%)	0.73±0.03	0.67±0.03	0.83±0.03	0.73±0.04	0.76±0.05	0.76±0.04	0.72±0.07	0.75±0.07	0.15
Jejunum length (cm)	13.17±0.32	13.49±0.29	14.21±0.30	13.27±0.35	13.57±0.48	14.38±0.33	13.68±0.62	12.89±0.62	0.09
Ileum W (%)	0.63±0.04	0.66±0.03	0.76±0.03	0.69±0.04	0.72±0.05	0.79±0.04	0.65±0.07	0.72±0.07	0.07
Ileum length (cm)	12.39±0.42^{b}	12.54±0.38^{b}	14.36±0.39^{a}	13.13±0.45^{ab}	13.95±0.63^{ab}	14.38±0.44^{a}	12.89±0.81^{ab}	12.86±0.81^{ab}	0.01
Small intestine W (%)	1.83±0.08^{b}	1.79±0.07^{b}	2.09±0.07^{a}	1.89±0.08^{ab}	2.01±0.11^{ab}	2.11±0.08^{a}	1.86±0.15^{ab}	1.94±0.15^{ab}	0.05
Small intestine Length (cm)	32.12±0.69^{c}	32.73±0.61^{bc}	35.36±0.65^{ab}	33.29±0.74^{bc}	34.88±1.03^{abc}	36.38±0.71^{a}	33.53±1.32^{abc}	32.53±1.32^{bc}	<0.01
Ratio of small intestine length to chick body W (%)	74.37±2.17	76.88±1.94	77.42±2.04	73.56±2.33	76.10±3.24	80.05±2.25	70.27±4.15	70.58±4.16	0.23

* Data are included least square means±SE. ^{a,b,c} Different superscripts within the same line means significant differences (P<0.05).

[1] All weight parameters are computed as their relative weight to chick weight for each individual bird. W stands for weight.

Table 4. The impact of L- methionine *in ovo* feeding on small intestine histology of day-old broiler chicks.

Traits *	Different levels of L- methionine *in ovo* feeding								P-value
	Control	Sham control	0.19%	0.38%	0.57%	0.76%	0.95%	1.14%	
Duodenum									
Villous height (µm)	223.55±14.18^{c}	216.05±16.08^{c}	250.34±21.27^{bc}	306.76±21.27^{b}	428.99±21.27^{a}	424.01±21.27^{a}	266.23±21.27^{bc}	238.19±21.27^{bc}	<0.01
Crypt depth (µm)	85.14±4.45^{ab}	93.78±5.04^{a}	75.76±6.67^{ab}	81.29±6.67^{ab}	79.53±6.67^{ab}	69.74±6.67^{ab}	63.52±6.67^{b}	78.69±6.67^{ab}	0.03
Villous height/crypt depth ratio (%)	2.68±0.27^{d}	2.39±0.30^{d}	3.31±0.40^{cd}	3.79±0.40^{bcd}	5.39±0.40^{ab}	6.30±0.40^{a}	4.35±0.40^{bc}	3.03±0.40^{cd}	<0.01
Villous thickness (µm)	58.50±3.41^{b}	57.06±3.86^{b}	66.25±5.11^{ab}	86.99±5.11^{a}	61.32±5.11^{b}	63.40±5.11^{b}	50.66±5.11^{b}	55.01±5.11^{b}	<0.01
Crypt diameter (µm)	21.47±1.46^{abc}	24.03±1.65^{ab}	24.39±2.18^{ab}	28.86±2.18^{a}	27.81±2.18^{a}	23.04±2.18^{abc}	17.93±2.18^{bc}	14.35±2.18^{c}	<0.01
Jejunum									
Villous height (µm)	111.76±9.14^{c}	116.18±10.37^{c}	259.87±13.72^{a}	143.68±13.72^{bc}	118.16±13.72^{c}	155.15±13.72^{bc}	202.19±13.72^{ab}	114.49±13.72^{c}	<0.01
Crypt depth (µm)	78.22±3.59^{b}	78.33±4.08^{b}	72.47±5.39^{bc}	63.17±5.39^{bc}	54.25±5.39^{c}	72.28±5.39^{bc}	75.76±5.39^{bc}	113.94±5.39^{a}	<0.01
Villous height/crypt depth ratio (%)	1.45±0.15^{de}	1.50±0.17^{cde}	3.59±0.23^{a}	2.37±0.23^{bc}	2.32±0.23^{bcd}	2.19±0.23^{bcd}	2.68±0.23^{ab}	1.01±0.23^{e}	<0.01
Villous thickness (µm)	44.65±4.32	44.27±4.90	58.49±6.49	58.17±6.49	50.35±6.49	43.60±6.49	57.66±6.49	49.88±6.49	0.32
Crypt diameter (µm)	22.03±1.07^{ab}	17.51±1.21^{b}	25.54±1.61^{a}	24.92±1.61^{a}	23.79±1.61^{ab}	25.09±1.61^{a}	22.16±1.61^{ab}	19.48±1.61^{ab}	<0.01
Ileum									
Villous height (µm)	70.17±5.26^{b}	70.09±5.96^{b}	74.35±7.89^{b}	71.54±7.89^{b}	74.07±7.89^{b}	114.74±7.89^{a}	88.13±7.89^{ab}	91.11±7.89^{ab}	<0.01
Crypt depth (µm)	90.38±4.08	80.56±4.63	87.06±6.12	84.39±6.12	83.26±6.12	97.62±6.12	87.93±6.12	95.52±6.12	0.37
Villous height/crypt depth ratio (%)	0.79±0.07	0.89±0.07	0.85±0.09	0.85±0.09	0.89±0.09	1.18±0.09	1.02±0.09	0.97±0.09	0.11
Villous thickness (µm)	42.58±3.31	42.55±3.75	47.54±4.97	51.00±4.97	47.06±4.97	50.66±4.97	51.09±4.97	42.12±4.97	0.59
Crypt diameter (µm)	19.37±1.24	18.55±1.41	25.53±1.86	19.49±1.86	19.76±1.86	23.02±1.86	21.89±1.86	17.96±1.86	0.07

* Data are included least square means±SE. ^{a,b,c,d} Different superscripts within the same line means significant differences (P<0.05).

Results

Based on the present results, hatchability was not significantly affected by L- methionine *in ovo* feeding (P= 0.06), though the highest tendency of hatchability was observed in 0.19% L- methionine treatment and the lowest tendency of hatchability was observed in 1.14% L- methionine treatment (Table 1).

Results indicated no effect of L- methionine *in ovo* feeding on chick weight, chick weight to egg weight ratio, and scalped carcass weight (P>0.05), but numerical increase in the L-methionine *in ovo* fed chicks were observed (Table 1). On the other hand, carcass efficiency and the weight of thigh, breast, and gizzard were all increased by L- methionine *in ovo* feeding treatments (P<0.05). Based on the results, the highest amount of carcass efficiency was observed in 0.19% and 0.76% L- methionine treatments, the highest thigh weight was observed in 0.19% L- methionine treatment, and the highest breast and gizzard weight were observed in 0.76% L- methionine treatment (Table 1). Pancreas weight was also tended to be higher in 0.57% and 0.76% L-methionine treatments (P=0.06), (Table 1). On the other hand, no effect of treatments on liver, heart, and proventriculus weight was observed (P>0.05), (Table 1).

In ovo feeding of L- methionine also increased thymus weight (P<0.01) and the highest amount was observed in 0.19% L- methionine *in ovo* feeding (Table 1). Meanwhile, bursa of Fabricius and spleen weight were not affected by L- methionine treatments (P>0.05), (Table 1).

Based on the results, L- methionine *in ovo* feeding significantly decreased serum glucose concentration (P<0.01), in which the lowest glucose concentration was observed in 1.14% L- methionine treatment (Table 2).

Blood urea nitrogen was also affected by L-methionine *in ovo* feeding treatments (P<0.05) and the lowest amount was observed in 0.19% L- methionine treatment (Table 2).

In ovo feeding of L- methionine significantly decreased serum triglyceride concentration (P<0.05) and the lowest amount was observed in 0.95% L- methionine group (Table 2).

No effect of L- methionine treatments was observed on serum total protein and cholesterol concentrations (P>0.05), (Table 2).

Duodenum length, ileum length, small intestine length, and small intestine weight were all affected by L- methionine *in ovo* feeding (P<0.05) and the highest amount was observed in 0.76% L- methionine treatment (Table 3). Based on the results, ileum weight (P=0.07) and jejunum length (P=0.09) tended to be higher in 0.76% L- methionine treatment (Table 3). Regardless, L- methionine treatments had no significant effect on duodenum weight, jejunum weight, and ratio of small intestine length to chick body weight (P>0.05), (Table 3).

Based on the duodenal histology results, L-methionine in ovo feeding significantly affected villous height, villous height/crypt depth ratio, villous thickness, crypt diameter, and crypt depth (P<0.05), (Table 4). Thus, the highest duodenum villous thickness and crypt diameter was observed in 0.38% L- methionine treatment, the highest duodenal villous height was observed in 0.57% L- methionine treatment, and the highest duodenal villous height/crypt depth ratio was observed in 0.76% L- methionine treatment (Table 4). On the other hand, the lowest duodenal crypt depth was observed in 0.95% L- methionine treatment (Table 4).

Results indicated an increasing effect of L-methionine treatments on jejunal villous height, villous height/crypt depth ratio, and crypt diameter (P<0.01), in which the highest amount was observed in 0.19% L- methionine *in ovo* feeding (Table 4). On the other hand, a decreasing effect of L- methionine treatments was observed on jejunal crypt depth (P<0.01) and the lowest amount was observed in 0.57% L- methionine group (Table 4). In contrast, L-methionine *in ovo* feeding did not affect jejunal villous thickness (P>0.05), (Table 4).

Results of the present study showed an increasing effect of L- methionine *in ovo* feeding on ileal villous height (P<0.01) and the highest amount was observed in 0.76% L-methionine treatment. Ileal crypt diameter tended to be higher in 0.19% L- methionine *in*

ovo feeding (P=0.07), (Table 4). Meanwhile, L- methionine treatments had no effect on ileal crypt depth, villous height/crypt depth ratio, and villous thickness (P>0.05), (Table 4).

Discussion

Present study indicated no effect of treatments on hatchability, though the highest hatchability was observed in 0.19% L- methionine group. In accordance with the present result, Mottaghitalab and Shafiymanesh (2015) reported no effect of *in ovo* injection of DL-methionine on hatchability, while Coskun et al. (2014) reported a decrease in hatchability with *in ovo* injection of DL- methionine (50 µl DL-methionine /1000 µl of 0.5% NaCl solution per egg). Ebrahimi et al. (2018b) with *in ovo* injection of different levels of DL- methionine (0.19, 0.38, 0.57, 0.76, 0.95, and 1.14% DL-methionine solved in 1ml distilled water) reported a decrease in hatchability rate, especially in 1.14% DL- methionine group. Coskun et al. (2018) reported a non-significant decrease in hatchability with *in ovo* injection of 2 mg DL- methionine /0.2 mL distilled water. Elwan et al. (2019) observed no significant difference in hatchability by the *in ovo* injection of the mixture of L-methionine and L- cysteine. Ghochkhani et al. (2017) with *in ovo* injection of different DL- methionine to L-lysine ratios reported no effect of treatments on hatchability. Furthermore, *in ovo* injection of zinc-methionine and nanozinc-methionine had no effect on hatchability (Razani et al. 2017). It seems that *in ovo* injection of DL-methionine (Coskun et al. 2014; 2018; Ebrahimi et al. 2018b) had more reverse effects on hatchability than *in ovo* injection of L- methionine (based on the results of the present study). This result may verify that chick embryo is more apt to use L- methionine compared with DL- methionine (Shen et al. 2015); therefore, L- methionine can support livability of embryo more than DL-methionine. Present results indicated that 1.14% L- methionine *in ovo* feeding numerically reduced hatchability which might be referred to amino acid imbalance or toxicity effect of this high level of L- methionine (Annongu et al. 2014; Mohammadrezaei et al. 2015; Ebrahimi et al. 2017). Observing different results by *in ovo* injection of methionine in different studies may relate to the use of different methods (*in ovo* injection into the air sac or amniotic fluid) or different days of *in ovo* injection (Coskun et al. 2014; Mottaghitalab and Shafiymanesh 2015; Ebrahimi et al. 2018b; Razani et al. 2017; Elwan et al. 2019).

Present results indicated that L- methionine *in ovo* feeding improved carcass efficiency, thigh, breast, and gizzard weight, while had no effect on chick weight and chick weight to egg weight ratio. In accordance with the present result, Ebrahimi et al. (2018b) reported that *in ovo* feeding of different levels of DL-methionine did not affect day-old chick weight, while improved carcass efficiency and thigh weight. Mottaghitalab and Shafiymanesh (2015) reported that *in ovo* injection of DL-methionine into amniotic fluid did not affect day-old chick weight. On the other hand, Coskun et al. (2014) reported an increasing effect of *in ovo* injection of DL- methionine on chick weight. Elwan et al. (2019) reported higher chick weight with *in ovo* injection of mixture of L-methionine (5.90 mg) and L-cysteine (3.40 mg). Also, Bhanja et al. (2012) reported that *in ovo* feeding of 25 mg DL-methionine improved chick weight to egg weight ratio and body weight of broiler chickens. Coskun et al. (2018) reported no effect of *in ovo* injection of 2 mg DL-methionine on chick weight to egg weight ratio and chicken weight (at the end of starter period). Razani et al. (2017) with *in ovo* injection of zinc-methionine and nanozinc-methionine reported higher chicks' weight at hatch and at 7 days post hatch. Ghochkhani et al. (2017) reported higher chick weight, carcass efficiency, and breast weight with *in ovo* injection of different DL- methionine to L-lysine ratios. The improving effect of *in ovo* feeding of L- methionine on carcass weight can be mediated through the direct role of methionine on protein synthesis (Annongu et al. 2014). Methionine may also improve growth by its role on the synthesis of

polyamines and L-carnitine (Bouyeh 2012; Ebrahimi et al. 2017). Carnitine with increasing in plasma insulin-like growth factor-I (IGF-I) concentration can stimulate protein synthesis and then, improve growth (Ebrahimi et al. 2017). Part of the improving effect of methionine on growth may also be mediated through stimulating of insulin (partly by increasing in pancreas weight as observed in the present study), thyroid hormones (T3 and T4), and IGF-I (Goodman 2010; Ebrahimi et al. 2017; Elwan et al. 2019). As optimum levels of thyroid hormones can stimulate growth of birds either before or after hatch (Ebrahimi et al. 2014); so, thyroid hormones may mediate part of improving effect of methionine on growth. Also, it was indicated that *in ovo* injection of mixture of L-methionine and L- cysteine increased total antioxidants capacity and glutathione in the serum, pectoral muscle, small intestine, liver, heart, and kidney of day-old broiler chicks (Elwan et al. 2019). Then, improving in antioxidant status of the embryos may accelerate their growth (Nazem et al. 2017). Moreover, observing lower blood urea nitrogen with *in ovo* feeding of L- methionine in the present study can be considered an indicator of higher protein synthesis, while lower protein degradation (Goodman 2010; Ebrahimi et al. 2014).

It was indicated that nutrient restrictions during early development postpones immune system maturation and growth (Bhanja et al. 2014). Then, *in ovo* feeding has beneficial effects on immune system (Ebrahimi et al. 2019). Results of the present study showed higher thymus weight with *in ovo* feeding of L-methionine. Though, our previous studies showed no effect of DL- methionine or DL-methionine to L-lysine ratios on immune system organs (Ebrahimi et al. 2018a,b). Higher bioavailability of L- methionine compared with DL- methionine (Shen et al. 2015) may be the reason of observing a better improvement in immune system organ (increase in thymus weight) in the present study. Bhanja et al. (2012) reported higher antibody titers with *in ovo* injection of DL-methionine, while no significant effect of DL-methionine on immune system organs was observed. Sigolo et al. (2019) reported no effect of feeding excess methionine (110% and 120% methionine based on Ross 308 recommendations) on lymphoid organs' weight (thymus, spleen, bursa of Fabricius) and antibody titers. The impact of methionine on immunity can be mediated through two mechanisms: 1- Methionine acts on biosynthesis of glutathione, which protects cells against oxidative stress (Bouyeh 2012; Elwan et al. 2019). 2- Methionine is necessary for synthesis of polyamines (spermine and spermidine), which stimulates cell divisions (Bouyeh 2012).

Results of the present study indicated a decrease in serum glucose, triglyceride, and blood urea nitrogen with *in ovo* feeding of L-methionine. Resemble to the present results, Ebrahimi et al. (2018b) with *in ovo* injection of DL- methionine reported lower glucose and blood urea nitrogen, but higher protein concentration. Bhanja et al. (2012) reported that *in ovo* injection of DL- methionine decreased plasma glucose, though increased protein concentration. Regardless, Sigolo et al. (2019) reported no effect of dietary surpluses of methionine on uric acid, total cholesterol, triglycerides, glucose, and total protein. It was indicated that an increase in the level of plasma amino acids stimulates insulin secretion from pancreas, in which insulin causes a reduction in blood glucose, triglycerides, and urea, while an increase in blood protein concentration (Goodman 2010; Ebrahimi et al. 2017). On the other hand, *in ovo* injection of amino acids reduces gluconeogenesis of fetus, which further causes low blood glucose level of hatchlings (Bhanja et al. 2012). Also, part of these effects of methionine on blood metabolites may be mediated by the role of methionine in biosynthesis of L-carnitine (Bouyeh 2012). Carnitine with inducing β-oxidation of fatty acids can cause a reduction in blood triglycerides (Ebrahimi et al. 2017). Carnitine can also stimulate IGF-I secretion, which further improves glucose uptake and reduces blood glucose levels (Goodman 2010;

Ebrahimi et al. 2017). In a study, *in ovo* injection of mixture of L-methionine and L-cysteine increased blood thyroid hormones (T_3 and T_4) and IGF-I expression in different tissues (pectoral muscle, heart, jejunum, and liver) of day-old broiler chicks (Elwan et al. 2019). Then, methionine directly increases IGF-I and protein synthesis; so, it decreases glucose and blood urea nitrogen levels (Goodman 2010; Elwan et al. 2019). Also, methionine by increasing in thyroid hormone secretion can accelerate the metabolism of lipids and then, it reduces triglyceride level (Goodman 2010; Elwan et al. 2019).

The present results showed an improving effect of L- methionine *in ovo* feeding on small intestine length and histology parameters of duodenum and jejunum (an increase in villous height, villous height/crypt depth ratio, and crypt diameter, while a decrease in crypt depth). Resemble to the present results, Ebrahimi et al. (2019) reported higher jejunal weight and higher villous height, crypt depth, villous height to crypt depth ratio, and villous thickness of small intestine (duodenum, jejunum, and ileum) with *in ovo* injections of DL- methionine. Bhanja et al. (2012) reported no effect of *in ovo* injection of DL- methionine on relative weight and length of small intestine. Coskun et al. (2018) reported no effect of *in ovo* injection of DL- methionine on villous length, villous width, and crypt depth. Mohammadrezaei et al. (2015) with *in ovo* injection of 20, 30, 40, and 50 mg DL-methionine (injection at d 4 of incubation and sampling of jejunum at d 18 of incubation) indicated an increasing effect of methionine (except for 50 mg methionine) on jejunal villous height and villous width. Also, it has been reported that *in ovo* injection of different DL- methionine to L- lysine ratios improved small intestinal weight, length, crypt depth, villous height, and villous height to crypt depth ratio (Ebrahimi et al. 2018a). Nazem et al. (2017) with *in ovo* injection of different methionine levels (20, 30, 40, or 50 mg DL-methionine) reported higher villous height, width, area and also higher enterocyte height, number of goblet cells, and muscle-layer thickness. In another study, *in ovo* injection of mixture of methionine (5.90 mg)/ cysteine (3.40 mg) improved villous height, width, surface area, crypt depth, and histological absorptive surface amplification of day-old broiler chick (Elwan et al. 2019). Razani et al. (2017) with intra amniotic injection of zinc-methionine and nanozinc-methionine indicated higher small intestine weight at d 1 and 7 of post hatch and more maltase and phosphatase activity of intestine at d 7 of post hatch. Shen et al. (2015) reported higher villi width and height, but lower crypt depth in broiler chickens fed on a diet containing 0.285% L- methionine compared with chickens fed on a diet containing 0.285% DL-methionine. Improvements in intestinal histomorphometric parameters (villi height, width, surface area, crypt depth) are indicators of improving intestinal maturity and its digestion and ingestion capacity (Hou and Tako 2018). The improving effect of L-methionine on intestinal growth and its histology may be mediated through several mechanisms. First, it was indicated that *in ovo* feeding per se can enhance intestinal growth and function as well as sooner adaptation to exogenous diets (Ebrahimi et al. 2017; Nazem et al. 2017; Razani et al. 2017; Elwan et al. 2019). Second, methionine can stimulate the secretion of insulin and IGF-I (Ebrahimi et al. 2017; Elwan et al. 2019), by which it can stimulate intestinal growth (Goodman 2010). It was indicated that higher gastrointestinal length and villous height causes higher absorption of nutrients (Ebrahimi et al. 2018a; Hou and Tako 2018). Also, a positive correlation between villous height and body weight gain and feed intake was observed (Ebrahimi et al. 2018a). Accordingly, *in ovo* feeding of L- methionine can improve hatchling growth by improving small intestine morphology parameters.

The overall results of this study indicated that *in ovo* feeding of 0.19% L- methionine (optimum level of L- methionine) improved or tended to improve hatchability, carcass efficiency, thigh, breast, thymus, small intestine length, and villous height/crypt depth

ratios of duodenum and jejunum, but reduced blood urea nitrogen in comparison with control groups. So, improving in carcass traits may accelerate the future growth of the chicks through several probable pathways: 1-an increase in satellite cell reservoir of the muscular tissues (Ball et al. 2007), 2-a reduction of muscular degradation by decreasing in blood urea nitrogen (Goodman 2010), 3- an improvement of digestion and absorption through increasing in intestinal growth and maturity (Ebrahimi et al. 2019; Elwan et al. 2019). Also, higher thymus weight may accelerate the future cellular immunity of chicks and increase the viability of the bird during rearing periods (Ebrahimi et al. 2019).

Conclusion

Results of the present study indicated improving effect of *in ovo* feeding of L-methionine on carcass weight indexes, blood metabolites, and small intestine morphology. Also, 0.19% L- methionine *in ovo* feeding considered an advisable level of *in ovo* feeding because of observing the highest hatchability rate in this group and its improving effect on immune system organs, small intestine growth, skeletal muscle development, and blood metabolites.

Conflict of interest statement

The authors have no conflict of interest to declare.

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اثر تغذیه درون‌تخم‌مرغی ال-متیونین بر صفات لاشه، ریخت‌شناسی روده‌کوچک و فراسنجه‌های خونی در جوجه‌های گوشتی یک‌روزه

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چکیده

زمینه مطالعاتی: متیونین به عنوان اولین اسید آمینه محدودکننده در طیور برای ساخت پروتئین و رشد ضروری است. در چندین مطالعه اثر تزریق درون‌تخم‌مرغی دی‌ال-متیونین بررسی شده است، اما در مورد تزریق درون‌تخم‌مرغی ال-متیونین گزارشی وجود ندارد. **هدف**: بنابراین هدف از این پژوهش بررسی اثر تغذیه درون‌تخم‌مرغی ال-متیونین بر وزن، صفات لاشه، ریخت‌شناسی روده‌کوچک و فراسنجه‌های خونی در جوجه‌های گوشتی یک روزه بود. **روش کار**: به منظور دستیابی به هدف این مطالعه، ۲۴۰ تخم‌مرغ مادر گوشتی (راس ۳۰۸) بر اساس طرح کاملا تصادفی با ۸ تیمار آزمایشی و ۳۰ تخم‌مرغ در هر تیمار استفاده شد. تیمارهای آزمایشی شامل ٦ سطح ال-متیونین (۰/۱۹، ۰/۳۸، ۰/۵۷، ۰/۷٦، ۰/۹۵ و ۱/۱٤ درصد ال- متیونین)، شم شاهد (تزریق آب مقطر) و شاهد (گروه بدون تزریق) بودند که در روز ۱٤ دوره جوجه‌کشی به درون مایع‌آمنیوتیک تزریق شدند. بعد از تفریخ، جوجه‌ها وزن کشی شدند، نمونه‌های خون جمع‌آوری شده و جوجه‌ها کشتار شدند تا صفات لاشه و ریخت‌شناسی روده‌کوچک بررسی شوند. سپس سرم نمونه‌های خونی جمع‌آوری شد و برای اندازه گیری غلظت متابولیت‌های خونی استفاده شد. **نتایج**: نتایج این پژوهش نشان داد تغذیه درون‌تخم‌مرغی ال-متیونین بازده لاشه و وزن ران، سینه، سنگدان و تیموس را افزایش داد ($P< ۰/۰۵$)، اما اثری بر جوجه‌درآوری نداشت ($P> ۰/۰۵$). همچنین، اثرهای کاهشی تیمارهای ال- میتونین بر گلوکز، تری گلیسرید و نیتروژن اوره‌ای خون مشاهده شد ($P< ۰/۰۵$). علاوه‌براین، تزریق درون‌تخم‌مرغی ال-متیونین موجب بهبود طول و وزن روده‌کوچک و فراسنجه‌های ریخت‌شناسی ژژنوم و دوازدهه شد (طول پرز، نسبت طول پرز به عمق کریپت، قطر کریپت و عمق کریپت)، ($P< ۰/۰۵$). **نتیجه گیری نهایی**: بر اساس نتایج این پژوهش، تغذیه درون‌تخم‌مرغی ۰/۱۹ درصد ال-متیونین اثر بهبود دهنده بر جوجه درآوری، صفات لاشه، فراسنجه‌های خونی، اندام‌های سیستم ایمنی و ریخت‌شناسی روده‌کوچک داشت؛ بنابراین ۰/۱۹ درصد ال-متیونین سطح قابل توصیه برای تغذیه درون‌تخم‌مرغی است.

واژگان کلیدی: ال- متیونین، تغذیه درون‌تخم‌مرغی، جوجه گوشتی، فراسنجه‌های خونی، روده کوچک