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Effects of *in ovo* Injection of Vitamins B_6 and B_{12} in Fertile Eggs Subjected to Ethanol Stress on Hatching Traits, Performance and Visceral Organs of Broiler Chicks Reared under Cold Stress Condition

Research Article

T. Momeneh[1] and M. Torki[1*]

[1] Department of Animal Science, College of Agriculture and Natural Resources, Razi University, Kermanshah, Iran

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*Correspondence E-mail: torki@razi.ac.ir

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ABSTRACT

Two subsequent experiments were conducted to evaluate the effects of *in ovo* injection of vitamin B_6 and B_{12} in fertile eggs subjected to ethanol (EtOH) stress on hatching traits (first), performance and visceral organs of broiler chicks under cold stress (second). A number of 510 fertile eggs were incubated. A number of 180 eggs were considered as controls (three subgroups as: not-injected, eggshell with a hole and distilled water-injected). A number of 110 eggs were injected with 25 µL of a 1:1 (v/v) mixture of EtOH 47.5% + distilled water. Eggs in two other groups were injected with 25 µL of a 1:1 (v/v) mixture of EtOH 47.5% + 100 µL of B_6 (n=110), 25 µL of a 1:1 (v/v) mixture of 47.5% EtOH + 1000 µL of B_{12} (n=110). A number of 240 one-day chicks, allocated to second experiment. Hatched chicks were divided into 4 treatments. Temperature was maintained 12 ˚C from 28 to 42 days of age. Hatchability percent ($P<0.05$) reduced by EtOH injected group. The lowest body weight at one day was observed in the EtOH injected group compared to other groups ($P<0.05$). No significant difference was detected in body weight gain and feed intake of chicks during 1-14 days of age between EtOH + B_6 and EtOH + B_{12} groups. There was no effect of treatment on feed conversion ratio and visceral organ weight ($P>0.05$). *In ovo* injection of vitamins B_6 and B_{12} alleviated EtOH-induced oxidative stress in chickens embryos. No significant difference was observed in the performance of the hatched birds in the cold conditions temperature ($P>0.05$).

KEY WORDS ethanol, broilers, performance, vitamins B.

INTRODUCTION

During the last century, the poultry industry underwent many changes that also affected the incubation industry (Hill, 2000). Stress is an important cause of reduced performance and increased susceptibility to disease. Under commercial industry conditions, chicks do not have access to feed until 48h after hatching (Dibner *et al*. 2008). Therefore, birds became more susceptible to pathogens (Dibner *et al*. 2008), their body weight decreased (0.18 g/h) (Bigot *et al*. 2003; Careghi *et al*. 2005), and restricted developments in critical tissues and organs, such as the intestine (Geyra *et al*. 2001; Dibner and Richards, 2004), immune system (Dibner *et al*. 2008) and pectoral muscle (Halevy *et al*. 2003; Moore *et al*. 2005). Several strategies have been proposed to improve performance during early development, such as feeding at the hatchery (Dibner *et al*. 1998; Careghi *et al*. 2005), and *in ovo* injection technology (Foye *et al*. 2006; Tako *et al*. 2004; Salami *et al*. 2014). *In ovo* injection decrease the need for enriched maternal diets

to achieve similar effect. It may also provide a peak absorption of exogenous nutrients and other agents by the embryo (Surai *et al*. 1999) and improved economically traits, such as weight gain, feed conversion, meat yield, and disease resistance (Ibrahim *et al*. 2012) that will lead to increased returns and efficiency for the industry (Schall, 2008). The use of *in ovo* injection is a novel solution in the research and industry application to provide developing embryos with nutrients compounds (Uni and Ferket, 2003) including amino acids (Ohta *et al*. 1999; Kadam *et al*. 2008), carbohydrates (Tako *et al*. 2004), vitamins (Gore and Qureshi, 1997; Ibrahim *et al*. 2012; Salami *et al*. 2014; Roman *et al*. 2012).

Exogenous EtOH and exogenous homocysteine (HoCys) are both teratogenic in chick embryos (Miller, 2004; Miller *et al*. 1996; Miller *et al*. 2000; Miller *et al*. 2003a; Miller *et al*. 2003b; Miller *et al*. 2006; Rosenquist *et al*. 1996) and reduced s-adenosylmethionine (SAM) levels, increased s-adenosyl homocysteine (SAH) levels, and decreased SAM/SAH ratios (Walcher and Miller, 2008; Kelsey *et al*. 2010). HoCys can be converted to methionine or cysteine by remethylation or trans-sulforation cycles with some enzymes (MS, SAM) and cofactors (B_6 and B_{12}). Insufficient vitamins B_{12}, B_6 and impairment in enzymes functions cause hyperhomocysteinemia (Taherianfard *et al*. 2013).

HoCys catabolism uses remethylation and transsulfuration pathways. In remethylation pathways, HoCys is remethylated back to methionine by using either betainehomocysteine methyl transferase, or methionine synthase, which uses 5-methyl tetrahydrofolate as the methyl donor (Selhub, 1999). In the transsulfuration pathway, HoCys is converted to cystathionine through the use of cystathionine β-synthase and cystathionine is ultimately converted into α-ketobutyrate, reduced glutathione (GSH), or taurine (Miller *et al*. 2011; Berning *et al*. 2013). Substances like vitamins B_{12} and B_6 can influence the methionine-homocysteine cycle and thus change concentrations of HoCys (Svingen *et al*. 2013).

The interactions of EtOH metabolism with the methionine–homocysteine cycle, together with the effects of vitamins B_6 and B_{12}, are not fully understood and more research trials are needed (Rajdl *et al*. 2016). There is no report studying on the *in ovo* injection of vitamin B_6, B_{12} and EtOH in broiler breeder eggs. Since EtOH increase HoCys levels within embryonic chick brains (Miller, 2004), the objectives of this study were to determine vitamin B_6 and B_{12} supplementation alleviates EtOH-induced oxidative stress in chick embryos and the performance of the hatched birds in the cold temperature. Therefore, this study was to investigate the effects of *in ovo* injection of vitamin B_6, B_{12} in fertile eggs subjected to EtOH stress on hatching traits, performance and visceral organs of broiler chicks reared under cold stress condition.

MATERIALS AND METHODS

All experimental protocols adhered to the guidelines of, and were approved by, the Animal Ethics Committee of Razi University (the ethic approval letter: AEC 23-2016).

Eggs incubation and injection

Two subsequent experiments were conducted to evaluate the effects of *in ovo* injection of vitamin B_6 and B_{12} in fertile eggs subjected to EtOH injection stress on hatching traits (first experiment), performance and visceral organs of broiler chicks (hatched in the first experiment) reared under cold stress condition (second experiment). In the first experiment, a number of 510 fertile eggs were incubated, weighed and distributed into 6 groups between 58 and 61 g obtained from broiler breeder. Fertile eggs (Ross 308) purchased from a local commercial hatchery. A number of 180 eggs were considered as control (three subgroups as:not-injected, eggs with a hole in eggshell, and eggs injected with distilled water). A number of 110 eggs were injected with 25 µL of a 1:1 (v/v) mixture of EtOH 47.5% + distilled water. Eggs in two other groups were injected with 25 µL of a 1:1 (v/v) mixture of EtOH 47.5% + 100 µL of B_6 (n=110), 25 µL of a 1:1 (v/v) mixture of 47.5% EtOH + 1000 µL of B_{12} (n=110) (Table 7). Each egg was numbered, weighed and incubated at 37.5 ˚C and 58% relative humidity up to 18 days with turned every 1 hours. On the first day of incubation, fertile eggs were candled and the width end of the eggs to be injected was sterilized with 70% EtOH. Injection was made according to the method described by Berning *et al*. (2013). For the last 3 days of incubation, the fertile eggs were transferred to the hatcher and kept there up to hatching. Temperature and relative humidity during hatch were 36.5 ˚C and 68% RH. Fertility was determined by candling after 7 days of age. Hatchability was recorded as percent of fertile eggs that hatched in each treatment by using the following equation:

Hatchability (%)= (number of eggs hatch/number of fertile eggs) × 100

Post-hatch growth performance

A number of 240 one-day chick (hatched chicks in the first experiment), were used for the second experiment. The chicks (Ross 308) were divided into 4 groups with 6 replications of 10 chicks each. Each group was housed separately in individual cages (Broiler house, Animal husbandary, farm, Razi University, Kermanshah, Iran).

Table 1 Composition (%) and calculated nutrient content of experimental diets

Ingredient	Starterperiod (1-14 day)	Growerperiod (14-28 day)	Finisher period (28-42 day)
Corn	55.00	58.00	61.05
Soybean meal	37.50	34.50	31.53
Soy oil	3.29	3.40	3.50
Lysine	0.20	0.18	0.13
DL- methionine	0.22	0.25	0.22
Threonine	0.10	0.07	0.3
Dicalcium phosphate	1.87	1.62	1.60
Carbonate	1.17	1.07	1.00
Salt	0.25	0.28	0.27
Sodium bicarbonate	0.15	0.13	0.15
Vitamin premix[1]	0.25	0.25	0.25
Mineral premix[2]	0.25	0.25	0.25
Nutrient content			
Metabolizable energy (ME) kcal/kg	2993	3056	3098
Crude protein (CP) %	21.2	20.1	18.94
Lys %	1.3	1.21	1.1
Met %	0.56	0.56	0.53
Cys %	0.35	0.34	0.32
Thr %	0.9	0.82	0.76
Ca %	0.94	0.84	0.8
Available Phos. %	0.45	0.41	0.4
Na %	0.16	0.16	0.16
Cl %	0.19	0.2	0.19
K %	0.92	0.87	0.82

[1] Provides per kg of diet: vitamin A (all-trans retinol acetate): 3600000 IU; Cholecalciferol: 800000 IU; vitamin E (DL-alpha-tocopheryl acetate): 7200 IU; vitamin K (menadion sodium bisulfate): 800 mg; Thiamine (thiamin mononitrate): 720 mg; Riboflavin: 2640 mg; Niacin: 12000 mg; Pyridoxin: 1200 mg; vitamin B_{12}: 6 mg; Calcium d-Pantothenate: 4000 mg; Folic acid: 400 mg; Biotin (d-biotin): 40 mg; Choline chloride (choline chloride): 100000 mg and Antioxidant (butylatedhydroxy toluene): 40000 mg.

[2] Provides per kg of diet: Manganese (MnO): 40000 mg; Zinc ($ZnSO_4.7H_2O$): 33880 mg; Iron ($FeSO_4.7H_2O$): 20000 mg; Copper ($CuSO_4.5H_2O$): 4000 mg; Iodine (KI): 400 mg and Se (Na_2SeO_3): 80 mg.

Table 2 Effects of *in ovo* injection of vitamins B_6, B_{12} and ethanol on body weight and weight gain from 1 to 42 days of age

Treatment*	Weight gain (g/day/chicken)				Body weight (g)			
	1-14 day	14-28 day	28-42 day	1-42 day	1 day	14 day	28 day	42 day
Control	31.43^{a}±0.81	63.18^{a}±2.44	95.98±6.14	63.52^{a}±2.62	44.96^{a}±0.51	491.96^{a}±14.19	1360.34^{a}±42.88	2760.40^{a}±120.91
Ethanol	24.06^{b}±0.89	56.81^{ab}±1.99	91.43±5.02	57.57^{ab}±2.39	42.37^{b}±0.57	374.66^{b}±11.59	1164.15^{bc}±35.01	2460.80^{ab}±110.38
Ethanol + B_6	22.93^{b}±0.81	52.87^{b}±2.19	92.36±7.09	50.90^{b}±2.39	41.73^{b}±0.51	361.38^{b}±12.69	1046.71^{c}±49.51	2210.50^{b}±110.38
Ethanol + B_{12}	24.95^{b}±0.89	59.11^{ab}±1.99	92.05±5.02	58.32^{ab}±2.39	44.60^{a}±0.57	377.09^{b}±11.59	1204.56^{b}±35.01	2558.21^{ab}±110.38
SEM	0.85	2.13	5.64	5.95	0.53	12.39	39.35	112.75
CV	6.89	8.47	13.24	10.21	2.61	7.19	7.15	10.99
P-value	< 0.0001	0.0371	NS	0.0172	0.0012	< 0.0001	0.0018	0.0143

* Control eggs were injected with 25 µL of distilled water; Control 1: sham control (no injection); Control 2: creating shell hole; Ethanol: 25 µL of a 1:1 (v/v) mixture of 47.5% EtOH + distilled water; Ethanol + B_6: 25 µL of a 1:1 (v/v) mixture of 47.5% EtOH + 100 µL of B_6 and Ethanol + B_{12}: 25 µL of a 1:1 (v/v) mixture of 47.5% EtOH + 1000 µL of B_{12}.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.
CV: coefficient of variation.
NS: non significant.

Table 3 Effects of *in ovo* injection of vitamins B_6, B_{12} and ethanol on feed intake (g/day/chicken) of broiler chicks from 1 to 42 days of age

Treatment*	Feed intake (g/day/chicken)			
	1-14 day	14-28 day	28-42 day	1-42 day
Control	41.97^{a}±1.58	112.54^{a}±5.72	188.74±9.93	115.59^{a}±4.75
Ethanol	34.21^{b}±1.77	95.85^{b}±4.67	180.82±8.11	105.59^{b}±4.34
Ethanol + B_6	31.16^{b}±1.58	87.77^{b}±5.12	169.04±11.47	90.19^{b}±4.34
Ethanol + B_{12}	35.39^{b}±1.77	94.08^{b}±4.67	176.71±8.11	102.42^{b}±4.34
SEM	1.67	4.99	9.11	4.43
CV	9.88	11.85	11.07	10.32
P-value	0.0021	0.0329	NS	0.0075

* Control eggs were injected with 25 µL of distilled water; Control 1: sham control (no injection); Control 2: creating shell hole; Ethanol: 25 µL of a 1:1 (v/v) mixture of 47.5% EtOH + distilled water; Ethanol + B_6: 25 µL of a 1:1 (v/v) mixture of 47.5% EtOH + 100 µL of B_6 and Ethanol + B_{12}: 25 µL of a 1:1 (v/v) mixture of 47.5% EtOH + 1000 µL of B_{12}.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.
CV: coefficient of variation.
NS: non significant.

The chicks were fed standard starter (ME, 3098 kcal/kg and CP, 18.94%), grower (ME, 3056 kcal/kg and CP, 20.1%) and finisher (ME, 2993 kcal/kg and CP, 21.2%) diets (Table 1).

Broiler chickens were used from 1 to 42 days of age to investigate *in ovo* injection of vitamin B_6 and B_{12} in fertile eggs subjected to EtOH injection stress on performance and visceral organs of broiler chicks (hatched in the first experiment) reared under cold stress condition (second experiment).

The hatched chicks were reared under normal temperature during the two weeks after hatch, and then the temperature was gradually reduced from days 14 of age and maintained 12 ˚C from 28 to 42 days of age. Light were on continuously for the first day post hatching, after which a 23 L:1 D lighting schedule was maintained for the duration of the experiment.

A basal diet was formulated for each of the three stages of growth: starter, grower and finisher. Birds were weighed as at hatch. The chicks were provided free access to feed and water during the experimental period. At 42 days of age, two birds were randomly selected from each replicate and sacrificed. Visceral organs were weighed and relative weights of organs were calculated. Care and management of the chicks were approved by the Animal Welfare Committee of the Razi University.

Statistical analysis

A completely randomized design was used with 6 replicates per treatment. The pen was used as an experimental unit. Data were analyzed according to general linear model (GLM) procedure of SAS (2008). Significant differences among treatment were considered at $P < 0.05$ by Duncans.

RESULTS AND DISCUSSION

Hatching traits

In the present study, the hatchability percentage (58.33 to 70.83%) was affected by injection of vitamin B_6 and B_{12} and EtOH (Table 8). The lowest hatchability percent was observed in the group of fertile eggs injected by EtOH ($P<0.05$). *In ovo* injection of B_6 (100 µL) and B_{12} (1000 µL) improved the egg hatchability. Sgavioli *et al.* (2016) reported hatchability was lower in the high-temperature groups (with and without vitamin C) than the control group. Ameenuddin *et al.* (1983) also reported the positive effect of *in ovo* injection of B_6 on hatchability. Improved hatchability (89.0%) compared to control (79.9%) was reported by Elsayed *et al.* (2010), who injected quail eggs with 120 µg B_6/egg before incubation. Based on the report by Elaroussi *et al.* (2003), the injection of quail eggs with 10 mg B_6/egg on day 7 of incubation resulted in improved hatchability (86.7%) compared to control (75.8%).

Table 4 Effects of *in ovo* injection of vitamins B_6, B_{12} and ethanol on feed conversion ratio (g/g) of broiler chicks from 1 to 42 days of age

Treatment*	Feed conversion ratio (g/g)			
	1-14 day	14-28 day	28-42 day	1-42 day
Control	1.33±0.08	1.78±0.08	1.96±0.09	1.82±0.09
Ethanol	1.43±0.09	1.68±0.06	2.00±0.07	1.84±0.08
Ethanol+B6	1.37±0.08	1.66±0.07	1.84±0.11	1.80±0.08
Ethanol+B12	1.43±0.09	1.60±0.06	1.92±0.07	1.76±0.08
SEM	0.09	0.08	0.07	0.08
CV	12.78	9.46	9.15	11.29
P-value	NS	NS	NS	NS

* Control eggs were injected with 25 µL of distilled water; Control 1: sham control (no injection); Control 2: creating shell hole; Ethanol: 25 µL of a 1:1 (v/v) mixture of 47.5% EtOH + distilled water; Ethanol + B_6: 25 µL of a 1:1 (v/v) mixture of 47.5% EtOH + 100 µL of B_6 and Ethanol + B_{12}: 25 µL of a 1:1 (v/v) mixture of 47.5% EtOH + 1000 µL of B_{12}.
The means within the same column with at least one common letter, do not have significant difference ($P>0.05$).
SEM: standard error of the means.
CV: coefficient of variation.
NS: non significant.

Table 5 Effects of *in ovo* injection of vitamins B_6, B_{12} and ethanol on visceral organ weight as percentage live weights of broiler at 42 days of age

Treatment*	Abdominal fat	Heart	Bursa	Spleen	Pancreas	Liver	Ileum	Jejunum	Duodenum
Control	0.97±0.12	0.63±0.07	0.23±0.03	0.08±0.01	0.23±0.03	2.21±0.27	1.19±0.15	1.28±0.16	0.63±0.08
Ethanol	1.06±0.15	0.69±0.10	0.23±0.04	0.09±0.01	0.26±0.04	2.42±0.36	1.31±0.19	1.40±0.21	0.69±0.09
Ethanol B_6	1.04±0.08	0.68±0.05	0.22±0.02	0.09±0.01	0.25±0.02	2.38±0.17	1.28±0.09	1.38±0.10	0.68±0.05
Ethanol + B_{12}	0.96±0.11	0.63±0.07	0.20±0.03	0.08±0.01	0.23±0.03	2.19±0.25	1.19±0.13	1.27±0.14	0.63±0.07
SEM	0.05	0.03	0.01	0.01	0.01	0.11	0.06	0.07	0.03
CV	12.09	11.91	12.93	12.90	11.78	12.02	12.00	12.00	12.02
P-value	NS	NS	NS	NS	NS	NS	NS	NS	NS

* Control eggs were injected with 25 µL of distilled water; Control 1: sham control (no injection); Control 2: creating shell hole; Ethanol: 25 µL of a 1:1 (v/v) mixture of 47.5% EtOH + distilled water; Ethanol + B_6: 25 µL of a 1:1 (v/v) mixture of 47.5% EtOH + 100 µL of B_6 and Ethanol + B_{12}: 25 µL of a 1:1 (v/v) mixture of 47.5% EtOH + 1000 µL of B_{12}.
The means within the same column with at least one common letter, do not have significant difference ($P>0.05$).
SEM: standard error of the means.
CV: coefficient of variation.
NS: non significant.

Improved hatchability of chicken eggs injected with 100 µg B_6/egg on day 14 of incubation were reported by Bhanja *et al*. (2007) and Ibrahim *et al*. (2012). Based on the report by Rajdl *et al*. (2016), vitamin B_{12} and vitamin B_6 supplementation did not lead to a statistically significant change in homocysteine.

Vitamin B_6 plays an important role in the synthesis and degradation of aspartate aminotransferase in the chicken embryo.

Pyridoxine has an important role in amino acid, carbohydrate, and fatty acid metabolism and also plays a major role in the energy-producing citric acid cycle (McDowell, 1989). Deficiency of vitamin B_6 led to early embryonic death and decreased IgM and IgG response to the antibody challenge (Blalock *et al*. 1984). Vitamin B_6, a water-soluble vitamin (Bender, 1999), resulted in embryonic growth retardation that led to its death and eventually poor hatchability (Ibrahim *et al*. 2012).

Also, deficiency of vitamin B_{12} caused decrease activity of Met synthase and failed to catalyze the folate-dependent remethylation HoCys to Met in brain (Min *et al*. 2005).

A significant decrease in the brain HoCys on the 15th day of the embryonic stage in eggs injected by EtOH and B vitamins was detected by Farahani *et al*. (2013). Injection of EtOH caused a significantly increased S-adenosyl methionine, which in turn activated methylation pathway in the hepatocytes in converting HoCys to Met (Carrasco *et al*. 2002).

Table 6 Effects of *in ovo* injection of vitamins B_6, B_{12} and ethanol on visceral organ weight as percentage live weights of broiler at 42 days of age

Treatment*	Back and neck	Wing	Legs	Breast	Live body	Lung
Control	19.16±2.36	6.63±0.82	24.85±3.06	38.97±4.79	2.81±0.37	0.54±0.07
Ethanol	20.92±3.06	7.23±1.06	27.13±3.97	42.54±6.23	2.59±0.41	0.59±0.09
Ethanol + B_6	20.59±1.49	7.12±0.52	26.71±1.94	41.89±3.05	2.59±0.20	0.58±0.04
Ethanol + B_{12}	18.97±2.19	6.56±0.75	24.61±2.84	38.59±4.46	2.83±0.31	0.54±0.06
SEM	0.96	0.33	1.24	1.95	0.14	0.03
CV	11.99	11.99	11.99	11.99	12.68	12.01
P-value	NS	NS	NS	NS	NS	NS

* Control eggs were injected with 25 µL of distilled water; Control 1: sham control (no injection); Control 2: creating shell hole; Ethanol: 25 µL of a 1:1 (v/v) mixture of 47.5% EtOH + distilled water; Ethanol + B_6: 25 µL of a 1:1 (v/v) mixture of 47.5% EtOH + 100 µL of B_6 and Ethanol + B_{12}: 25 µL of a 1:1 (v/v) mixture of 47.5% EtOH + 1000 µL of B_{12}.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.
CV: coefficient of variation.
NS: non significant.

Table 7 *In ovo* injection of experimental treatments

Treatment*	Water	Ethanol	B_6	B_{12}
Control (1)	-	-	-	-
Control (2)	-	-	-	-
Control	✓	-	-	-
Ethanol	✓	✓	-	-
Ethanol + B_6	✓	✓	✓	-
Ethanol + B_{12}	✓	✓	-	✓

* Control eggs were injected with 25 µL of distilled water; Control 1: sham control (no injection); Control 2: creating shell hole; Ethanol: 25 µL of a 1:1 (v/v) mixture of 47.5% EtOH + distilled water; Ethanol + B_6: 25 µL of a 1:1 (v/v) mixture of 47.5% EtOH + 100 µL of B_6 and Ethanol + B_{12}: 25 µL of a 1:1 (v/v) mixture of 47.5% EtOH + 1000 µL of B_{12}.

Table 8 Effects of *in ovo* injection of vitamins B_6, B_{12} and ethanol on egg weight, body weight, chick weigh/egg weight ratio and hatchability (%)

Treatment*	Egg weight (g)	Body weight (g)	Body weight/egg weight (g/g)	Hatch (%)
Control	61.43±1.60	44.96^{a}±0.47	0.74±0.02	83.33^{a}±3.41
Control (1)	60.37±1.60	45.13^{a}±0.43	0.75±0.02	80.03^{ab}±3.41
Control (2)	59.01±1.60	44.96^{a}±0.43	0.76±0.02	74.90^{abc}±3.41
Ethanol	61.34±1.60	42.36^{b}±0.52	0.71±0.03	58.33^{d}±3.41
Ethanol + B_6	59.44±1.60	41.73^{b}±0.47	0.70±0.02	68.75^{c}±3.41
Ethanol + B_{12}	58.73±1.60	44.60^{a}±0.52	0.76±0.03	70.83^{c}±3.41
SEM	1.60	0.47	0.03	3.41
CV	6.52	2.37	6.59	11.51
P-value	NS	< 0.0001	NS	0.0002

* Control eggs were injected with 25 µL of distilled water; Control 1: sham control (no injection); Control 2: creating shell hole; Ethanol: 25 µL of a 1:1 (v/v) mixture of 47.5% EtOH + distilled water; Ethanol + B_6: 25 µL of a 1:1 (v/v) mixture of 47.5% EtOH + 100 µL of B_6 and Ethanol + B_{12}: 25 µL of a 1:1 (v/v) mixture of 47.5% EtOH + 1000 µL of B_{12}.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.
CV: coefficient of variation.
NS: non significant.

Based on the study by Berning *et al*. (2013), exogenous EtOH caused elevated hepatic HoCys levels in the brain and liver of chick embryo via change in the methylation pathway and folate concentration (Carrasco *et al*. 2002; Taherianfard *et al*. 2013; Berning *et al*. 2013).

Performance and visceral organs

Average feed intake, weight gain, feed conversion, and body weight of the chickens are provided in Tables 2, 3 and 4. Present results showed that, weight gain during 1-14 days of age control group were significantly increased ($P<0.05$). There was no significant difference in weight gain and feed intake of chicks during 28 to 42 days of age between groups under cold temperature ($P>0.05$). Feed intake of control group was significantly increased during 1-14 days and 14-28 days of age ($P<0.05$). Feed conversion ratio during 1 to 42 days was not significantly affected by treatments ($P>0.05$). The effects of treatments on the relative weight of visceral organ are presented at Tables 5 and 6. Sajid *et al*. (2007) reported non significant difference ($P>0.05$) in the live weight of broilers received different doses of EtOH (40%). Present results showed that, chickens reared under the cold temperature presented higher weight gain and feed intake. This result is consistent with the results observed by Sgavioli *et al*. (2016), and appears to be related to body temperature maintenance. Al-Daraji *et al*. (2012) reported no significant feed intake differences between Japanese quails injected or not *in ovo* with L-arginine, but the *in ovo* injection of L-arginine resulted in better feed conversion ratio. Similarly, no significant effect of the *in ovo* injection of broiler embryos with selected substances on feed conversion ratio were detected. In contrast, Salmanzadeh *et al*. (2012) reported that the broilers submitted to *in ovo* injection of glucose presented better feed conversion ratio during the rearing period than the control group. Feed intake and feed conversion ratio were not affected by supplemental propolis in broiler (Mahmoud *et al*. 2013). Salmanzadeh *et al*. (2016) reported that the effect of *in ovo* feeding of glutamine caused lower hatchability than in the control group. Chickens from the effect of *in ovo* feeding of glutamine showed better weight gain and feed conversion ratio (0-42 days of age), when compared to chickens hatched from control and sham groups. In addition, carcass weights and relative weights of breast, thigh and gizzard were also markedly increased in chickens treated *in ovo* with glutamine, whereas heart, liver, abdominal fat, intestine, pancreas and spleen were not significantly altered. In addition, Bleich *et al*. (2003) showed that EtOH gradually decreases the liver's abilityin folate storage. Bree *et al*. (2001) also explained an inverse relation between folate and HoCys level (Bree *et al*. 2001). Farahani *et al*. (2013) reported folate deficiency as a probable reason for brain HoCys accumulation. Relatively lower weight gain in the broilers in the present study could be partly due to dose-dependent effect of EtOH, which was in turn responsible for low feed intake. It has been demonstrated that EtOH increased serum level of HoCys (Sakuto and Suzuki, 2005; Bleich *et al*. 2000; Bleich *et al*. 2003; Bleich *et al*. 2005). This reaction is catalyzed by a pyridoxial-5′-phosphate (vitamin B_6) containing enzyme, cystathionine β-synthase (Kelsey *et al*. 2010). It is known that EtOH and its metabolites influence several key conversion enzymes of Met-HoCys (Desilva *et al*. 1998). EtOH-induced toxicity reduced in the mouse fetus due to maternal supplementation of B_{12} (Xu *et al*. 2006; Kelsey *et al*. 2010).

CONCLUSION

Based on the obtained results of the present study, *in ovo* injection of EtOH reduced performance and hatchability percentage. Also, no significant difference was observed between the performance of the hatched birds in the cold conditions temperature. In addition, *in ovo* injection of vitamins B_6 and B_{12} alleviated EtOH-induced oxidative stress in chick embryos.

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