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Physiological and Histological Responses of Broiler Chicks to *in ovo* Injection with Folic Acid or L-Carnitine During Embryogenesis

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Abstract: The aim of the present study was to evaluate the effect of *in ovo* injection with folic acid or L-Carnitine (L-Car) on performance, some blood biochemical and muscles traits of posthatch chicks. Eggs were obtained from broiler breeder stock and divided into four similar groups at 14 day of incubation. The 1st group received no treatment (negative control) while the 2nd was subjected to *in ovo* injection of 0.1 ml distilled water/ egg as solvent (S) into the air cell (positive control). The third one was given *in ovo* folic acid at a concentration of 1 mg / egg and the fourth group was *in ovo* injected with 1mg / egg of L- Car. The present findings revealed that chicks hatched from eggs treated with folic acid or L-Car has significantly better productive performance (higher LBW, BWG and better FCR) compared with untreated one. Body fat% and intramuscular fat% (on dry matter basis) were significantly reduced with treatments, especially for L- Car. Broiler chicks whose eggs were treated with folic acid or L-Car had lower plasma concentration of total lipid, cholesterol, triglycerides and LDL. Both treatments elevated LDH activity and plasma levels of T_3, T_4 and IGF-I at 6 weeks of age. Folic acid and L-Car induce myocytes hypertrophy which in turn enhances carcass % and breast muscle yield.

Key words: *In ovo* · Embryo · Folic Acid · Carnitine · Muscle · Broiler

INTRODUCTION

Poultry diets have a high percentage of cereal grains that are poorly contain amino acids essential for better performance. Thus, the livestock researchers and producers tended to examine new feed additives that can compensate for this shortage and consequently, benefit the poultry health and production. This presents considerable opportunities for the use of a recent physiological feed additive; Folic acid or L-carnitine, that have gained interest in recent years as potential physiological additives for enhancing domestic animal production because of their tremendous metabolic functions. Hence, plants and plant-based feedstuffs generally contain very little folic acid or carnitine, compared with animal sourcas,that caused a barrier for optimum metabolic requirement [1].

In ovo feeding of supplemental nutrients may help to overcome the constraint of limited egg nutrients. Rapid growth, high energy requirement and the limited embryonic synthesis of L-Carnitine may make supplementation of L-carnitine beneficial to chicken embryos. Moreover, several studies illustrated that, fortification of fertile eggs with different nutrients, i.e. vitamins, L-Carnitine before incubation was reported as promised tool to improve carcass quality by increasing lean to fat percentage and enhance different performance traits and immunity of broilers [2-6]. L-carnitine (β-hydroxy γ-trimethylaminobutyrate) is an amino acid derivative compound exists in cells of all living organism in various amount [7]. It is synthesized *in vivo* from the amino acids lysine and methionine that act as precursors [8]. The endogenous synthesis of L-carnitine requires the ferrous ions and a number of vitamins;

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ascorbate, folate, niacin and pyridoxine [9]. The principal function of L-carnitine is the transport of long-chain fatty acids from the cytosol into mitochondria for β-oxidation; consequently it sustains the supply of energy [10, 11].

Folic acid is a synthetic form, well-known as a one of the water-soluble vitamin B-complex group. Moreover, it is needed to produce DNA as cells multiply functions as coenzymes in single-carbon transfer in the synthesis and metabolism of nucleic and amino acids [12, 13]. Moreover, Grieshop *et al.* [14] declared in swine fed supplemental folic acid that, it was seemed to participate in the proliferation of immunoglobulin producing cells eliciting greater immune responses. Earlier study by Samuel [15] showed that chicken fibroblast proliferation can be strongly stimulated by the addition of folic acid, at greater than physiological concentrations, to a plasma-containing medium. The effect of *in ovo* folic acid on hatching traits was studied by Robel [16] they found that folic acid fortification did not increase the hatchability percentage of fertile turkey hens, nor it did influence the posthatch body weight. Because of the scarcity of available information, concerning the effect of folic acid on broiler performance as well as the histological changes of muscles, the present study was conducted to elucidate the influence of *in ovo* injection with folic acid or L-Carnitine on performance, some blood parameters, plasma hormones and muscular growth of broiler chicks.

MATERIALS AND METHODS

Experimental Procedures: A total of 480 eggs with an average weight of 67 g were obtained from a commercial broiler breeder flock (Cobb-500) at 55 weeks of age. At day 14 of incubation period, eggs were randomly divided into four groups each of 120 eggs; each includes three replicates of 40 eggs. All eggs were normally incubated at 37.6°C and 65% RH in an automatic incubator. The first group, was considered as negative control, given no treatment (C) while the second group, was considered as positive control, subjected to *in ovo* injection of 0.1 ml distilled water/ egg as solvent (S) into the air cell. The third group was injected into air cell with folic acid (FA) at a concentration of 1 mg /egg and the fourth group was injected into air cell with L-Carnitine (L-Car) at a concentration of 1 mg /egg. Egg injection procedure was carried out at day 14 of embryonic development. Thus, each egg was cleaned and the large top of the egg (location of air cell) was disinfected by ethyl alcohol. The point site of injection was punctured by hard and thin stylus and the tested material was injected into the air cell of each egg by using graded insulin syringe (1 ml) and the punctured site was sealed with non-toxic glue stick. At the 18th day of incubation, all eggs were transferred to the hatcher and kept till hatching at 37.2°C and 70% RH.

The hatched chicks from the four groups were fed *ad libitum* on commercial starter (1 to 28 d) and finisher (28 to 42 d) crumbled diets. Chicks were individually weighed at hatch and then at the end of the experimental period (6 wks) and their live body weights (LBW) were recorded. The body weight gain (BWG), feed consumption (FC) and feed conversion ratio (FCR) was calculated.

Biochemical Analysis: At the end of the experimental period, 6 weeks of age, 5 chicks were randomly taken and slaughtered by severing the carotid arteries and jugular veins. Blood samples were collected, during their exsanguinations in heparinized tube and centrifuged at 4000 rpm for 15 min., plasma samples were assigned for the determination of total protein, albumin, total lipids, triglycerides, cholesterol, LDL-cholesterol and HDL-cholesterol using available commercial kits. Globulin was calculated by subtraction of plasma albumin from total protein. The radioimmunoassay (RIA) method was used for the determination of triiodothyronine (T_3), thyroxine (T_4) and insulin like growth factor-I (IGF-I). Plasma T_3 and T_4 were determined by RIA technique using commercial RIA kits (Immunotech, Beckman Coult. Company) as reported by Britton *et al.* [17]. Insulin-Like Growth Factors (IGF) was determined with commercial kits (Gammacoat, kits, clinical assay, Cambridge, Medical Diagnostics, Boston, MA) as reported by Houston and Neill [18]. The major and minor pectoralis muscles were also dissected and weighed. All weights were expressed as percentages of live body weight. The intramuscular content of fat in both major pectoralis and thigh muscles was measured according to the AOAC [19].

Histological and Cytological Measurements: At 6 weeks, a representative sample (0.5×0.5 cm) from the left major muscle pectoralis of the chicks (three from each of the four groups) was carefully dissected and immediately fixed in adequate volume of 10% formalin solution. The paraffin technique has been used according to Abd El-Hamid [20]. Thin sections (4-5 micron) were cut and mounted on glass slides (three sections/sample/slide), then stained with the ordinary hematoxyline and eosin

stain procedures. Muscle sections were examined by using a trinocular light microscope (Labomed, L_X 400. Labo America, Inc. USA) supplied with a computerized digital camera (iVU 3000) endorsed with software (ProgRes® CapturePro 2.707, 2010) which measures the image element dimensions in Pxl unit. The Pxl unit was calibrated to the micrometer unit at different magnification power using a micrometric ruler (PZO- WARS ZAWA- Made in Poland). The myonuclei number in three pectoral muscles sections obtained from 6 weeks old chicks were counted in nine different fields (3/section) for all treatments, at 10x. Large and small diameters of 100 myocytes/ slide/ treatment were recorded to obtain some cytometric and histometric indexes according to Radu-Rusu *et al*. [21] using the following formula,

- Mean thickness (*MT*; µm) = (*LD* + *SD*) / 2
- Shape index ratio (*SI*) = *LD* / *SD*
- Cross section area (*SA*; µm^2) = (*LD* × *SD* × π) / 4

Where:

LD = Myocyte large diameter
SD = Myocyte small diameter
π = 3.1416

Statistical Analysis: Data were subjected to one-way analysis of variance using the General Linear Models (GLM) procedure of SAS [22]. Duncan's Multiple Range Test [23] was used to separate means when separation was relevant. All Percentage data were subjected to arcsine transformation of the square root before statistically reanalyzed however, the actual percentage means are presented. Statistical significance was accepted at a probability level of 0.05 ($P \leq 0.05$).

RESULTS AND DISCUSSION

Results presented in Table 1, showed that chicks delivered from eggs which injected with folic acid had significantly improved live body weight (LBW), body weight gain (BWG), feed consumption and feed conversion ratio (FCR) followed by L-Car treatment compared with chicks of control treatment. These effects of folic acid injection may be related, in part, to thyroid hormone (T_3) activity and (or) to the positive effects of folic acid in improving nutrients utilization. The valuable effects of pre incubation injection of FA may be interpreted through its recent definition as an antioxidant and (or) biological additive to eggs before incubation. In accordance with the present findings, concerning FA inclusion on hatchling body weights, Khalifah and Shahein [24] reported that body weight of newly hatched chicks was significantly increased when their dams fed on diets contained supplemental FA. Also, the improvement in body weight with L-Car could be due to an increase in the efficiency of fatty acids oxidation, which subsequently led to an improved utilization of dietary nitrogen thereafter [11]. However, Zahi *et al.* [25] reported that *in ovo* injection of L-Car into fertile eggs at 17 or 18 d of incubation did not affect LBW.

The effect of *in ovo* folic acid or L-Car injection on carcass %, major and minor pectoralis %, Body fat % and intramuscular fat% of breast and thigh are present in Table 2.

In ovo injection with folic acid or L-Carnitine treatment significantly increased the carcass percent of broilers at marketing age (6 weeks) compared with the control groups, this may be due to the anabolic effect of both treatments on muscle building. These results are in close agreement with those reported by Robel, who used folic acid and L-Carnitine treatments in turkey,

Table 1: Effect of *in ovo* injection by FA or L-Car during embryogenesis on productive performance of broiler chicks.

	Item				
	Live body weight				
Treatment	Hatch	42 day	Weight gain	Feed consumption	Feed conversion ratio
C	0.049^{b}±0.009	2.081^{d} ±0.02	2.039^{c} ±0.02	4.205^{a} ± 0.04	1.863^{b}±0.002
S	0.048^{b}± 0.008	2.163^{c}±0.01	2.118^{b}±0.001	4.137^{a}±0.05	1.963^{a}±0.03
FA	0.050^{a} ± 0.002	2.305^{a}±0.02	2.261^{a}±0.02	3.783^{c} ±0.25	1.803^{b}±0.02
L-Car	0.049^{b}± 0.002	2.232^{b}±0.05	2.168^{b}±0.001	3.897^{b}±0.03	1.857^{b}±0.001
Significance	*	***	***	***	**

C, (negative control); S, (positive control); FA, (folic acid); L-Car, (L-Carnitine)

* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, NS= non-significant.

a, b and c Means within columns with no common superscripts differ significantly

Table 2: Effect of *in ovo* injection by FA or L-Car during embryogenesis on breast muscles relative weight, body fat and intramuscular fat of broiler chicks.

	Item					
Treatment	carcass%	Major pectoralis%	Minor pectoralis%	Body fat%	Intramuscular Breast Fat%	Intramuscular Thigh fat%
C	71.78^{b}±0.22	7.58^{a}±0.30	1.78^{b}±0.01	2.76^{a}±0.04	3.03^{a} ±0.02	4.09^{a} ±0.07
S	71.13^{b}±0.37	7.18^{b}±0.11	1.69^{c}±0.04	2.87 ^{a}±0.15	2.35^{b}±0.04	4.11^{a}±0.15
FA	73.56^{a}±0.64	7.81^{a}±0.47	1.89^{a}±0.08	2.72^{a}±0.07	2.34^{b}±0.07	3.99 ^{ab} ±0.22
L-Car	72.97^{a}±0.26	7.68^{a}±0.12	1.87^{a}±0.03	2.26^{b}±0.50	2.28^{b} ±0.14	3.69^{b} ±0.09
Significance	**	*	***	***	***	*

C, (negative control); S, (positive control); FA, (folic acid); L-Car, (L-Carnitine)

* P≤ 0.05, ** P≤ 0.01, *** P≤ 0.001, NS= non-significant.

a, b and c Means within columns with no common superscripts differ significantly

Table 3: Effect of *in ovo* injection by FA or L-Car during embryogenesis on plasma total proteins, albumin, globulin, total lipids, triglycerides, cholesterol, HDL and LDL of broiler chicks.

	Item							
Treatment	Total proteins (g/dl)	Albumin (g/dl)	Globulin (g/dl)	Total lipids (mg/dl)	Triglycerides (mg/dl)	Cholesterol (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
C	6.46^{c} ± 0.08	3.15^{b} ± 0.09	3.33^{c} ± 0.09	769.98±12.48	76.95^{a}±1.37	154.90±1.72	58.14±0.95	84.94^{a} ±1.27
S	6.46^{c} ± 0.09	3.09^{c} ± 0.05	3.35^{c} ± 0.05	765.90±13.14	75.56^{ab}±2.51	156.08 ±1.09	57.45±1.23	82.36^{b}±2.06
FA	6.76^{b} ± 0.08	3.26^{a} ± 0.07	3.53^{b} ± 0.04	759.85±11.51	74.22^{bc} ±1.21	157.83±2.33	58.14±1.02	82.09^{b}±1.32
L-Car	7.09^{a} ± 0.03	3.24^{a} ± 0.04	3.78 ± 0.06	758.86±9.98	73.93^{c} ±1.65	153.91 ±1.68	58.18±1.08	80.27^{b}±1.12
Significance	**	**	**	NS	*	NS	NS	*

C, (negative control); S, (positive control); FA, (folic acid); L-Car, (L-Carnitine)

* P≤ 0.01,** P≤ 0.001, NS= non-significant.

a, b and c Means within columns with no common superscripts differ significantly

Gursu *et al.*, who studied effects of vitamin C and folic acid supplementation on serum paraoxonase activity and metabolites induced by heat stress *in vivo* and Sarica, *et al*., who studied the effect of dietary l-carnitine supplementation in Japanese quail [16, 26, 27].

The results shown in Table 2 indicated that *in ovo* injection with folic acid or L-Car caused a significant increase in the major and minor breast muscles weight of chicks at 6 weeks of age. This effect may be due to that the positive effects of folic acid and L-Car on breast muscles yield reflect their beneficial use in improving muscles yield of broilers. The previous results are in agreement with those reported by Abdel-Fattah and Shourrap, Lourens *et al.* and Piestun *et al.* [5, 13, 28, 29].

The results in Table 2 indicated that *in ovo* injection with L-Car was significantly reduced the total body fat (abdominal, gizzard and neck fats), Intramuscular Breast Fat (% from dry matter) and Intramuscular Thigh fat (% from dry matter) followed by folic acid compared with the control groups. This effect may be related to the action of L-Carnitine on energy metabolism, as a hypolipidemic drug which induces fatty acids catabolism and splitting ATP energy. This result agreed with those obtained by Abdel-Fattah and Shourrap, Xu *et al*. and Uni *et al*. [5, 13, 30, 31].

Blood Constituents: Results in Table 3, showed that in ovo injection with L-Car significantly increased plasma total proteins, albumin and globulin of chicks at 6 weeks followed by folic acid compared with chicks of control groups (C and S). These results may be attributed to the useful effect of L-car in sparing both methionine and lysine (its precursors) for protein biosynthesis and other cellular functions moreover, optimizing the balance between essential and nonessential amino acids [27] Besides, the suggested immunostimulator role of L-carnitine, induces the production of immunoglobulins, consequently elevated the total globulin [6].

The results concerning the effects of *in ovo* injection of L-Car and folic acid on plasma total lipids and triglycerides are presented in Table 3. Data indicated that L-Car treatment insignificantly decreased plasma levels of total lipids while significantly decreased the level of triglycerides at 6 weeks of age followed by folic acid compared with the control groups.

In ovo injection with folic acid or L-Car during embryogenesis did not affect the level of HDL while L-Car insignificantly decreased cholesterol and LDL level at 6 weeks comparable to the other groups. The beneficial effect of L-Car administration in reducing the plasma concentration of triglycerides, cholesterol and LDL

Table 4: Effect of *in ovo* injection by FA or L-Car during embryogenesis on plasma LDH, T_3, T_4 and IGF of broiler chicks.

	Item			
Treatment	LDH	T3	T4	IGF
C	406.53^{b}±2.23	4.72^{d}±0.19	22.99^{d}±0.96	31.19^{d}±1.14
S	405.43^{b}±8.71	4.89^{c}±0.95	23.23^{c}±0.86	32.13^{c}±1.12
FA	485.96^{a}±9.02	5.22^{a}±0.28	24.60^{a}±0.57	46.67^{a}±1.19
L-Car	460.62^{a}±4.64	5.16^{b}±0.49	24.26^{b}±0.14	39.93^{b}±0.97
Significance	*	*	*	*

C, (negative control); S, (positive control); FA, (folic acid); L-Car, (L-Carnitine)

* P≤ 0.01,, NS= non-significant.

a, b,c and d Means within columns with no common superscripts differ significantly

could be associated with the effect of L-Car in decreasing the lipoprotein lipase (LPL) activity. Xu *et al.* [30] reported that LPL catalyses the conversion of triglycerides to glycerol and fatty acids. Moreover with the reduction of LPL activity, it increases hydrolysis of LDL, which plays a major role in regulating the deposition of body fat content and thus, minimizes subcutaneous fat deposition.

Our findings concerning the effect of L-Car on plasma total lipids and its fractions are in harmony with the results of Keralapurath *et al.* and Xu *et al.* [11, 30]. Moreover, Arslan [4] demonstrated that L-Car has been reported as a hypolipidemic drug, able to reduce the circulating concentration of cholesterol, triglycerides, free fatty acids, phospholipids and very low density lipoproteins (VLDL) and to increase the concentration of high and intermediate density lipoproteins (HDL and IDL).

Table 4, illustrates the effect of *in ovo* injection with folic acid or L-Car of broilers eggs on Lactate dehydrogenase (LDH) activity, triiodothyronine (T_3), thyroxine (T_4) and IGF-1 concentrations.

Lactate dehydrogenase (LDH) was significantly increased by *in ovo* injection with L-Car or folic acid compared with the control groups. This may be related to its role in reoxidation of NADH via lactic formation which allows glycolysis to proceeds. The higher values of LDH associated with *in ovo* L-Car or folic acid are in close agreement with previous studies [5, 13, 26].

Plasma T_3 level significantly increased by *in ovo* injection with folic acid followed by L- car compared with both control groups, similar trend was observed for the T_4 level. This increase was also shown by Abdel-Fattah and Shourrap [5, 13] in broiler chicks hatched from eggs *in ovo* administered with L-carnitine and folic acid. In partial accordance with our results, Janssens *et al.* [32] who reported that, in spite of the absence of statistical significance, an increase in the plasma level of T_3 and T_4 in pigeon fed supplemental L-Car was observed.

It is clear from Table 4, that *in ovo* injection of folic acid or L-Car increased the plasma levels of insulin like growth factor-I (IGF-I). This result may be due to that folic acid induced the synthesis of IGF's via different routs including its effect on DNA and RNA synthesis. Similarly, *in ovo* administration of L-Carnitine increased IGF's of broilers chicks [5, 13, 33].

Cytological and Histological Changes: The effect of *in ovo* injection by FA or L-Car during embryogenesis on myocytes numbers, thickness and cross-section area of the myocytes are presented in Table 5 and Fig. 1.

The number of myocytes (per microscopic field) of the pectoral muscle was significantly reduced in folic acid and L-Car but L-Car chicks did not differ from the control groups. It appears, however, that FA and L-Car injection may induce the developmental growth pattern of myocytes via a hypertrophy process instead of the hyperplasia that observed in the control sections (Fig. 1).This may explain the increased breast muscle yield at 6 weeks this was also observed by Abdel-Fattah and Shourrap [5, 13].

This holds true as the large diameter (LD) and small diameter (SD) of myocytes were markedly increased due to folic acid then L-car treatment, comparable to the control groups (Table 5).

Data of mean thickness (MT) of myocytes showed similar aforementioned trend of LD and SD with the folic acid and L-Car treatment. Moreover, the same trend was observed in the shape index values (the ratio between large and small diameter of myocytes). The cross section area (SA) of myocytes was significantly increased by *in ovo* injection with folic acid or L-car treatment.

From the histological and cytological measurements and observation of pectoral muscles, it is concluded that in ovo injection of folic acid and L-Car could affect myonuclei division during embryonic myogenesis.

Table 5: Effect of *in ovo* injection by FA or L-Car during embryogenesis on myocytes numbers, thickness and cross-section area of the myocytes.

	Item					
Treatment	Myocytes number/ field	Large diameter (*LD* µm)	Small diameter (*Sd* µm)	Mean thickness (D $\bar{x}$ µm)	Shape index (*SI*) (*LD*/*Sd*ratio)	Cross section area (*SA*µm^2)
C	263.06 ^{a} ±4.80	51.86^{c} ±0.72	40.80^{c} ±0.62	46.33^{c}±0.67	1.31^{c}±0.03	1692.67^{c} ±48.87
S	255.26^{ab}±5.94	55.52^{bc}±1.24	39.06^{c}±0.87	47.55^{c}±0.53	1.39^{bc}±0.02	1715.33^{c}±41.44
FA	227.34^{b}±5.11	89.14^{a}± 3.63	53.10^{a} ±1.22	71.64^{a} ±2.37	1.71^{a}±0.04	3918.90^{a} ±50.93
L-Car	250.04^{ab}±3.62	61.65^{b}±2.82	45.65^{b}±1.92	53.59^{b}±2.41	1.46^{b}±0.06	2246.47^{b}±85.32
Significance	*	**	**	**	**	**

C, (negative control); S, (positive control); FA, (folic acid); L-Car, (L-Carnitine)

* P≤ 0.05, ** P≤ 0.01, NS= non-significant.

a, b and c Means within columns with no common superscripts differ significantly

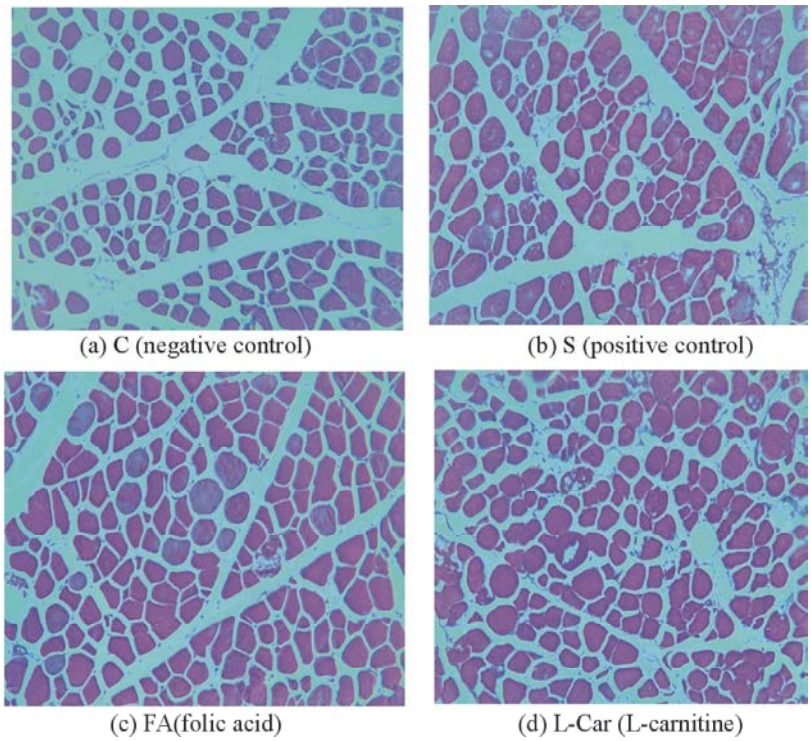

Fig 1: Transverse section through breast muscle from chicks at 6 weeks.

Hence, this beneficial effect is reflected in promoting the final live body weight and carcass (%) of chicks at 6 weeks of age. This finding could support the suggestion that pre incubation FA or L-Car fortification of eggs is a practical tool for enhancing the productive performance of broiler chicks.

CONCLUSION

It could be concluded from the present findings that, egg fortification with folic acid or L-Car could improve the post hatch performance of broiler chicks due to modulating the physiological response. Thereby, the meat yield was improved at the marketing age through their effect on muscle myogenesis during embryogenesis and development during post hatch period. Therefore, further researches are needed to shed more light on the application of these procedures.

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