BMC Veterinary Research

RESEARCH

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Intra-amniotic injection of L-carnitine reveals age-related effects on tissue total antioxidant status and increases plasma testosterone levels in male broiler chicks

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Abstract

Background L-carnitine, derived from methionine and lysine, is present in plasma and tissues as free active carnitine in poultry. L-carnitine (L-car) plays a role in mitochondrial metabolism by enhancing β-oxidation and as an antioxidant molecule in the mitochondria. L-car synthesis is limited during embryonic development of birds. Therefore, the aim of this study was to investigate the influence of intra-amniotic injection of L-car on total antioxidant status (TAS) in tissues, plasma testosterone level and developmental parameters in male broiler chicks at the posthatching stage. The 360 eggs used as experimental material were divided into 3 groups: a non-injection group (control group) and in ovo (IO) injection groups, which were pure water (PW) group, or pure water + L-carnitine (PW + L-car) group. The 2.5 mg of L-car in 500 µl of PW was injected to fluid of the amnion membrane at day 18 of incubation. The total antioxidant status in the yolk before incubation and yolk membranes at day 19 of incubation and at hatching was analysed. Blood plasma testosterone and liver TAS levels were measured at hatching and at days 3 and 7 of age. Organ development, morphological characteristics and relative asymmetry (RA) of bilateral lengths were quantified at the same measuring days.

Results The TAS levels of yolk and residual yolk membranes decreased in comparison to the TAS in the initial yolk. The TAS in yolk membrane was higher than in residual yolk membrane in the IOPW + L-car group. Male chicks in the IOL-car group had the lowest TAS level in the liver at day 3 of age. Plasma testosterone level was significantly found higher in the IOPW + L-car group than in the other groups at all the ages. The chick development was not affected by IOPW + L-car injection. At hatching, IOPW + L-car group had shorter beak, face and middle toe lengths and lower RA of face length than control group at day 7 of age.

Conclusion In conclusion, IOPW + L-car injection reveals age-related effects on tissue TAS levels and increases testosterone level interacted with the formation of the hypothalamic-pituitary–testicular axis of male broiler chicks at post-hatching stage.

Keywords L-carnitine, In ovo injection, Amnion fluid, Total antioxidant status, Testosterone, Broiler chick

Background

Bioactive compounds and egg composition in relation to the maternal environment influence embryogenesis in broiler breeders. In response to increased nutritional requirements of the developing embryo, the diet of broiler breeders is being manipulated to improve the

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nutrient composition of the egg [1], but these manipulations in diet of broiler breeders are not economical. The main reason for this is the transfer and accumulation of nutrients into developing follicles in the ovaries by maternal plasma over a long period of time. Therefore, early feeding practices in comparison to applications based on maternal nutrients are attracting attention as more effective applications in a short period of time. In ovo (IO) feeding is one of the most effective of early feeding applications. IO feeding includes the injection of various nutrients to fluid of the amnion membrane of the embryo, which begins to consume nutrients orally and this starts functional absorption in the gut [2, 3] by days 17 and 18 of embryonic age. Therefore, the amniotic membrane of the embryo is the injection site of amino acids that have important roles in the gastrointestinal system, antioxidant status, hatching performance, and development and growth of the embryo/chick [3]. An example, IO feeding of lysine improves the antioxidant status, and combination of lysine and cysteine improves the chick growth performance in broiler at posthatching stage [4]. Another example, IO feeding of methionine improves digestive enzyme activities, nutrient transport, jejunal antioxidant status, intestinal development and jejunal morphology in geese [5]. Furthermore, the highest dose of IO L-carnitine (L-car) feeding (injected into the amnion membrane; 0.5, 2.0 or 8.0 mg / egg on day 18 of incubation), derived from methionine and lysine, was reported to significantly increase hatchability [6].

L-carnitine is present in plasma and tissues in a free form of active carnitine in poultry. L-carnitine plays a role in mitochondrial metabolism, which enhances β-oxidation, thereby providing energy production through the catabolism of fatty acid molecules. Thus, it is noteworthy that L-car acts as an antioxidant molecule by suppressing the production of oxidant molecules in the mitochondria [7]. It was concluded that concentrations of delta-tocopherol and alpha-tocotrienol in the residual yolk membrane of 3 day-old male broiler chicks were increased by IO injection of L-car on the day 17 of incubation [8]. This conclusion is based on the molecular mechanisms of interaction between L-car and vitamin E on the antioxidant system, which are related to their respective contributions to antioxidant defence in different ways [9].

The results of numerous studies have indicated that the primary biological effects of L-car on embryonic development are as follows: maintenance of energy production, promotion of lipid metabolism through β-oxidation, modulation of glucose metabolism, reduction of free radical formation, protection of cells against oxidative stress, enhancement of respiratory chain enzyme activities, and improvement of antioxidant enzymes activity [10]. L-carnitine is synthesised from methionine and lysine in the availability of the Fe^{2+} and vitamin niacin, vitamin C and vitamin B_6. L-car synthesis has been detected in avian embryos, but this synthesis is reported to be limited during embryonic development [10]. Although the site of L-car synthesis is muscle, due to the presence of lysine in muscle, the liver is the main site of its synthesis. It has been demonstrated that 25 mg L-car in kg diet deposited in the egg yolk was transmitted to the liver mediated absorbed yolk membrane by day 18 of embryonic age [11]. It was also found that L-car levels increased in the yolk membrane and liver at day 18 of embryonic age with affected yolk membrane β-oxidation [11]. Furthermore, L-car injection into the yolk membrane increases liver glycogen level and glycogen index at 500 µg of its dosage, and plasma growth factor-1 level and chick weight at 25–500 µg of its dosage [12]. L-carnitine levels above 10 µmol (1.612 mg/egg) may enhance the development of the newly hatched chick [13]. It may also affect morphological features related to development. Therefore, the study aimed to examine effects of intra amniotic IOL-car injection on TAS in tissues of embryo and chick, plasma testosterone level and chick development of male broilers during one weekly age after hatching.

Methods

The experimental procedures of the study were carried out in accordance with the guidelines of the 'European Union Directive 2010/63/EU on the Protection of Animals Used for Scientific Purposes'. According to this legislation, embryos and chicks used as experimental material for measurements were subjected to cervical dislocation as stated in [14]. Following the completion of the experiment, the surviving chicks were delivered to Van Yuzuncu Yil University The Animal Husbandry Application and Research Centre for rearing. These delivered animals were not euthanised, as they were not subjected to any subsequent treatment and were not used as sample material. A total of 360 eggs with an average weight of 61 ± 0.78 g were obtained from a commercial breeder of the Ross genotype at 38 weeks of age. After gender determination, newly hatched female and male chicks were reared to 7 days of age.

Experimental design and in ovo procedure

The eggs, which were stored at a temperature of 16 °C and a relative humidity level of 80% for a period of seven days, were weighed and incubated. The ambient temperature and relative humidity in the incubator (CIMUKA T series 1280 capasity, Ankara, Türkiye) was at 37.7 °C and at 60% from day 1 to day 18, and at 37.4 °C and at 70% from day 19 to day 21. A total of 120 eggs per group were placed in eight trays of 15 eggs each, with eight repetitions, in

order to ensure greater uniformity of environmental conditions in the incubator. The eggs divided into 3 groups were non—injection group (control group) and in ovo (IO) injection groups, in which pure water (PW), or pure water + L-carnitine (PW + L-car) was injected into fluid of the embryo' amniotic membrane. The control group eggs had no injection, while 2.5 mg L-car (L-carnitine for synthesis, CAS No: 541—15–1, Merck KGaA, Darmstadt, Germany) dissolved in 500 µl PW (per egg) was injected into fluid of amniotic membrane on the day 18 of incubation. The 500 µl of PW without the addition of L- car was injected into the same site of embryos of the same age. The injection site on eggs sterilised with 70% ethanol was punctured with a 22 G needle. Prepared solutions were injected into the amniotic membrane with a 26 G, 18 mm needle using a semi—automatic injector. A paraffin wax was used to close the hole in the egg and then the embryonated eggs were immediately placed in the hatching trays. At hatching, hatched chicks were transferred to rearing pens as 3 replicates (24 chicks / replicate / group). From day 1 to day 7, the chicks were fed a commercial diet containing 23% protein and 3100 kcal/kg.

Examined characteristics

Total antioxidant status in the yolk of egg at the initial of incubation, and in the yolk membrane of the embryo and in the residual yolk membrane of the chick was analysed in the control and injection groups. In order to determine the TAS level on the 19th day of incubation, after the fertility control was performed in 10 eggs in each group, the yolk membrane of the embryo was taken from the broken egg and stored at −20 until the analysis.

In each group, measurements were made on male chicks allowed to dry for 4 h after hatching. Liver TAS and plasma testosterone levels of 10 randomly selected male chicks from each group (10 samples/group/day) were measured on days 0, 3 and 7, which were 3 different chick ages in the early developmental period after hatching.

Chick developmental parameters

The weight of the chick, yolk membrane, pectoral muscle, liver, brain, gizzard, heart, brain, lung, bursa of fabricius and spleen were quantified at measuring days. Relative organ weights were calculated as follows:

Relative weight = absolute weight/chick weight*100.

Chick length was measured between the tip of the upper beak and the last digit of the middle toe of the chick lying on its right side. Head diameter, lengths of beak, the left and right of face, shank and middle toe were quantified as suggested by [15]. Relative asymmetry (RA) of the measured bilateral lengths was calculated by RA = (|Left measurement (L) −Right measurement| (R)/[(L + R)/2]) × 100 [15]. The body mass index was determined by dividing the chick's weight by its length squared (g/cm^2).

Hatching results

Fertility rate (%) and hatchability rate (%), following formulas were calculated: Fertility rate: number of fertile eggs / total number of eggs * 100; Hatchability: number of hatched chicks / number of fertile eggs * 100. Embryonic deaths were shown as a ratio of deceased embryos to fertilised eggs, classified according to the age range of death.

Post-hatch growth performance

Growth performance was evaluated in terms of chick weight, feed consumption, feed conversion ratio and mortality rate. Chick weight was determined at 0, 3 and 7 days of chick age. Feed consumption was determined by calculating the difference between the offered and remaining feed amounts. The ratio of the amount of feed consumed to chick weight was calculated as feed conversion ratio. To calculate the mortality rate, the number of dead chicks was recorded for each group.

Total antioxidant status analysis in the tissues

Tissue samples were extracted according to the ELISA (enzyme-linked immunosorbent assay) procedure. Weighed tissues were treated with 9 × potassium chloride (KCL) and homogenised for 1 min. The homogenates were resuspended in 1 ml KCL. Tissue extracts were stored at −20 °C until analysis.

Plasma testosterone assay

Blood samples were centrifuged (SIGMA 3 30 K) at 3750 rpm for 10 min at 4 °C. Testosterone level of plasma was analysed by ELISA analysis as described in [14].

ELISA prosedure

Chicken-specific testosterone hormone (Chicken—specific Testosterone ELISA kit, Rel Assay Diagnostics, Gaziantep, Türkiye) and TAS (Chicken-specific Total Antioxidant Status ELISA kit, Rel Assay Diagnostics Gaziantep, Türkiye) kits were purchased from a commercial company. Testosterone hormone and TAS concentrations were analysed using ELISA microplate reader (Biotek EL × 800 ELISA reader, California, CA, USA) at a wavelength of 450 nm. Determined absorbance values were calculated by the standard curve equation [14, 16] from the values plotted on the graph with the standard concentrations. These calculations were used to determine TAS concentrations in tissues and testosterone hormone concentrations in blood plasma.

Statistical analysis

Data were analysed using a general linear model procedure, with main effects (group and age) and interaction between them using two-way analysis of variance (ANOVA) for TAS and plasma testosterone levels, and with group effect using one-way ANOVA for chick developmental and bilateral morphological traits. Incubation results and growth performance traits were analysed using the chi-squared test on the SAS package program [17]. Student's t-test was used to evaluate the differences between the means ($P < 0.05$) for chick developmental parameters, plasma testosterone concentration, and tissue TAS levels.

Results

Hatching results and Post-hatch growth performance

The effect of IO L-car administration on the hatching results and post-hatch growth performance traits was presented in Table 1. The effect of IO L-car application on hatchability and embryonic mortality was not significant. In ovo L-car administration had no effect post-hatch growth performance traits (Table 1).

Total antioxidant status in the yolk and yolk membranes

The total antioxidant status in the yolk membrane of embryos was influenced by IO L-car administration (Table 2). The total antioxidant status in the yolk membrane of the IOPWL-car injection group increased, but the IOPWL-car injection group did not differ from the control and IOPW groups. The total antioxidant levels of the yolk membrane on the 19 day of embryonic age and the residual yolk membrane at hatching decreased similarly compared to the initial TAS level of the yolk (Table 2; Fig. 1). However, the TAS in the yolk membrane was found to be higher than that in the residual yolk membrane in the IOPWL-car injection group (Fig. 1).

Table 2 Total antioxidant status (TAS) in the egg yolk, yolk memrane, resudial yolk membrane and liver, and plasma testosterone concentration of male broiler chicks exposed to in ovo L-carnitine injection

	Yolk/Yolk mebrane TAS, nmol/l	Liver TAS mmol/l	Plasma testosterone pg/ml
In ovo L-carnitine injection (Group)			
C	0.57 ± 0.04^{A}	1.66 ± 0.04	67.39 ± 3.77^{B}
IOPW	0.41 ± 0.04^{B}	1.60 ± 0.03	77.67 ± 4.45^{AB}
IOPW + L–car	0.45 ± 0.05^{AB}	1.61 ± 0.03	88.90 ± 4.26^{A}
P value	0.053	0.881	0.003
Age, day			
Embryonic age 0[1]	1.75 ± 0.06^{A}	–	–
19	0.54 ± 0.04^{B}	–	–
Chick age 0[2]	0.42 ± 0.03^{B}	1.73 ± 0.02^{A}	63.13 ± 3.94^{B}
3	-	1.59 ± 0.03^{B}	89.41 ± 4.25^{A}
7	-	1.53 ± 0.04^{B}	81.44 ± 4.33^{A}
P value	*P* < .001	0.008	*P* < 001
Group*Age			
P value	0.051	0.043	0.027

[AB] Different superscript means differ significantly $p < 0.05$. *C* Control, non—injection group, *IOPW* In ovo pure water group, *IOPW + L—car* In ovo L—carnitine group added pure water. [1]At onset of incubation, [2]At hatching, or at day 21 of incubation

Table 1 The effect of in ovo L-carnitine injection on hatching performance and post-hatch growth performance

	IO L-car treatment			
	C	IOPW	IOPW + L-car	Prob > χ^2
Hatching performance				
Fertility rate, %	94.45	95.52	95.18	0.841
Hatchability %	87.73	88.14	86.89	0.445
Early embryonic dead, from 1 to 5 day %	5.53	5.12	5.63	0.663
Mid embryonic dead, from 6 to 14 day %	2.23	1.19	1.32	0.291
Late embryonic dead, from 15 to 21 day %	2.10	2.54	2.29	0.354
Non-pipped dead at hatching, %	2.44	3.01	3.89	0.286
Total embryonic mortality %	12.27	11.86	13.11	0.445
Post-hatch growth performance				
Mortality rate %	-	1.55	-	0.312
Body weight gain, from 0 to 7 day, g	25.28	23.71	24.57	0.245
Feed consumption, from 0 to 7 day, g	22.11B	20.83C	25.28A	0.089
Feed conversion rate, from 0 to 7 day	1.45	1.20	1.46	0.176

[ABC] Different superscript means differ significantly $p < 0.05$

C: Control, non—injection group; IOPW: In ovo pure water injection group; IOPW + L-car: In ovo L-carnitine injection group added pure water

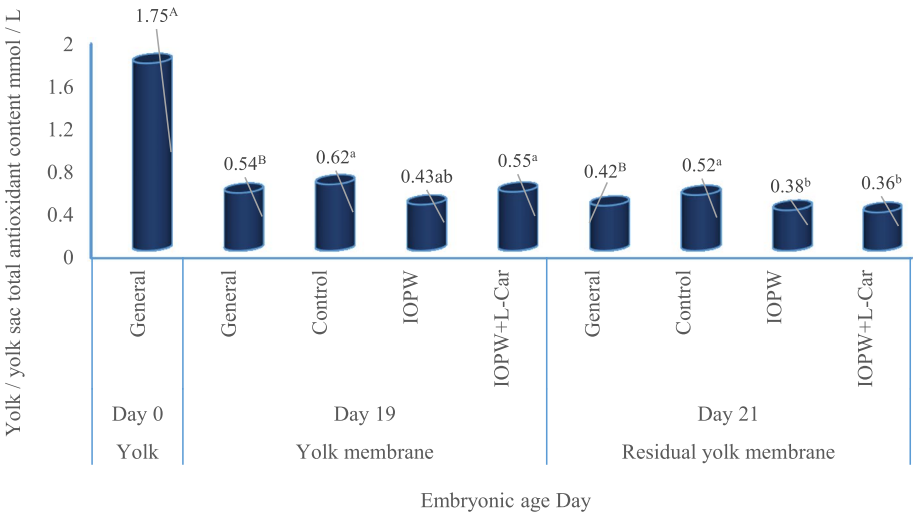

Fig. 1 Egg yolk total antioxidant status (TAS) at the initial of incubation, and the effect of interaction between in ovo L-car administration and embryo/chick age on TAS in the yolk membrane of embryo and residual yolk membrane of male chicks. Control: Non - injection group; IOPW: In ovo pure water injection group; IOPW + L-car: In ovo L-carnitine injection group added pure water. [AB, ab]Different superscript means differ significantly $P<0.05$.

The total antioxidant status in the residual yolk membrane of chicks in the control group was higher than in the injection groups. These conclusions led to the interaction between IOPWL-car administration and embryonic age being accepted as significant (Fig. 1).

Total antioxidant status in the liver

In ovo L-car administration had no effect on TAS concentration of the liver in chicks (Table 2). The chick age influenced the tissue TAS levels. The highest TAS level were found in the liver tissue of day-old chicks, whereas TAS levels decreased in the liver tissues of 3- and 7-day-old chicks. (Table 2). A significant interaction was found between IO L-car administration and chick age (Fig. 2). The reason for this interaction was that male chicks in the IOPWL-car group had the lowest liver TAS level compared with 3 day-old male chicks in the control group (Fig. 2).

Plasma testosterone concentration

Plasma testosterone concentration was significantly affected by IOPWL-car administration ($p=0.053$), chick age ($p<0.001$) and their interaction ($p=0.051$). The highest plasma testosterone level was found in the

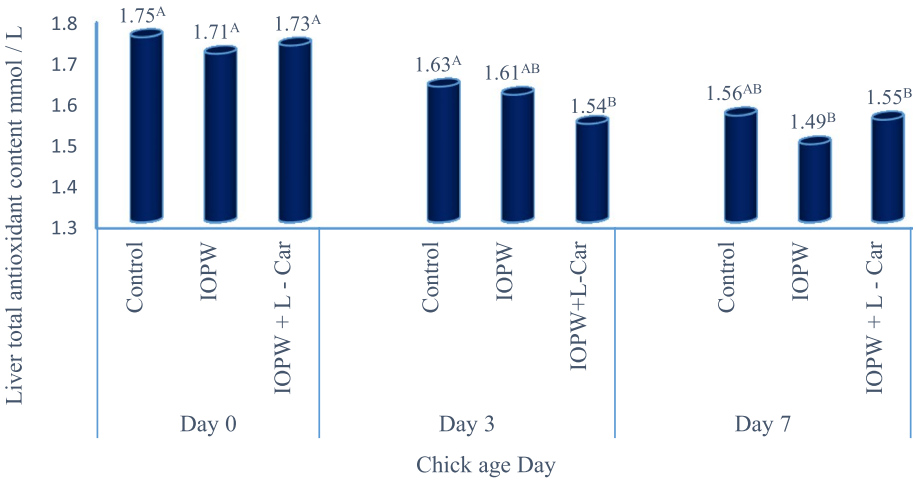

Fig. 2 The effect of interaction between in ovo L-car administration and chick age on total antioxidant status (TAS) in the liver of male chicks. Control: Non - injection group; IOPW: In ovo pure water injection group; IOPW + L-car: In ovo L- carnitine injection group added pure water. [AB]Different superscript means differ significantly $P<0.05$.

IOPWL-car group, whereas the lowest plasma testosterone level was in the control group, but both were not different from the IOPW group (Table 2). Plasma testosterone concentration of male chicks at days 3 and 7 of chick age showed a similar change compared to hatching, this result was due to significant chick age effect (Table 2). Plasma testosterone levels of chicks in the IOPWL-car injection group were significantly increased compared with the control and IOPW groups at all ages examined (Fig. 3). However, plasma testosterone levels of male chicks in the control group were found to be significantly lower at hatching, whereas male chicks in the control group had similar plasma testosterone levels at 3 and 7 days of age, reaching approximately twice the level at hatching. A similar result was found for the IOPW group (Fig. 3).

Chick developmental parameters

The developmental parameters of the chicks are presented in Table 3. There was no influence of IOL-car on chick weight at different chick ages. IOPWL-car injection group did not affect the weights of yolk membrane and examined organs during the early development stage after hatching. While the chick length at hatching and at day 7th of age was unchanged with L-car treatment, it was significantly declined in the injection groups at day 3 of age (Table 4). At hatching, beak, face and middle toe lengths were significantly lower in the IOPWL-car group than control group. On the 3 day of age, there was a similar decrease in the length of the middle toe of chicks in the IOL-car group. IOL-car application did not affect on the other morphological traits examined at the corresponding ages (Table 4). Bilateral traits were unchanged with IO L-car administration, but RA of the face length was significantly lower in the IOPWL-car group than that of the control at day 7 of chick age (Table 4).

Discussion

The hypothesis of this study was whether IO L-car injection into the fluid of the amniotic membrane of the embryo stimulates TAS in tissues during the periods of late embryogenesis and early chick development. The conclusions of the current study indicated that the antioxidant status of the tissues more effectively activated by increased TAS in the yolk membrane of embryos injected with L-car. This activation reflects L-car's protective effects on the developing embryo's antioxidant defence system. These protective effects of L-car may be due to enzymatic antioxidants and non-enzymatic antioxidants in cells mobilised to the tissues from the yolk membrane of the embryo and also it improved the antioxidant defence system to facilitate hatching, a physiological process caused by oxidative stress. The TAS level in the residual yolk membrane of newly hatched chick treated with L-car injection may have pointed out a decrease in total concentrations of lipid-soluble vitamins, carotenoids and selenium in the residual yolk membrane at hatching. This result has shown a more effective utilisation of these antioxidants to protect tissues from lipid peroxidation and a greater transfer of antioxidants to the tissues via the yolk membrane of chick. In embryos injected with L-car, it was reported that L-car induced increased energy by catabolising fatty acids in the yolk

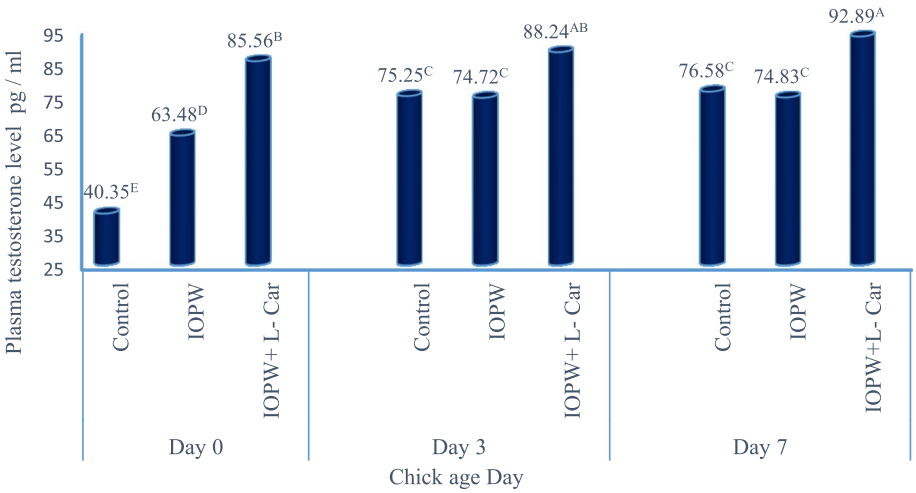

Fig. 3 The effect of interaction between in ovo L-car administration and chick age on plasma testosterone concentration of male chicks. Control: Non - injection group; IOPW: In ovo pure water injection group; IOPW + L-car: In ovo L-carnitine injection group added pure water. ^{ABCDE}Different superscript means differ significantly $P<0.05$.

Table 3 Chick development parameters of male broiler chicks exposed to in ovo L-carnitine injection

	Chick weight g	Yolk sac g	Liver %	Lung %	Heart %	Bursa fabricius %	Spleen %	Brain %	Breast muscle %	Gizzard %
Day, 0										
C	47.92 ± 0.85	6.32 ± 0.47	2.55 ± 0.07	0.923 ± 0.08	0.635 ± 0.01	0.121 ± 0.01	0.03 ± 0.01	1.85 ± 0.16	1.86 ± 0.07	5.49 ± 0.25
IOPW	45.62 ± 0.85	5.14 ± 0.47	2.58 ± 0.07	0.937 ± 0.08	0.660 ± 0.01	0.105 ± 0.01	0.05 ± 0.01	2.01 ± 0.16	1.69 ± 0.07	5.45 ± 0.25
IOPW + L-car	47.08 ± 0.85	5.64 ± 0.47	2.5] ± 0.07	0.967 ± 0.08	0.613 ± 0.01	0.09 ± 0.01	0.04 ± 0.01	1.80 ± 0.16	1.85 ± 0.07	5.00 ± 0.25
P value	0.177	0.225	0.947	0.928	0.151	0.206	0.286	0.653	0.228	0.3372
Day, 3										
C	84.38 ± 3.19	0.77 ± 0.23	3.90 ± 0.13	0.88 ± 0.04	0.62 ± 0.03	0.12 ± 0.01	0.06 ± 0.01	1.13 ± 0.05	3.53 ± 0.27	5.29 ± 0.15
IOPW	73.39 ± 4.52	0.79 ± 0.37	3.73 ± 0.18	0.84 ± 0.06	0.64 ± 0.05	0.08 ± 0.01	0.04 ± 0.01	1.30 ± 0.07	2.59 ± 0.38	5.28 ± 0.22
IOPW + L-car	81.77 ± 4.52	1.59 ± 0.33	3.97 ± 0.18	0.86 ± 0.07	0.54 ± 0.05	0.13 ± 0.01	0.05 ± 0.01	1.19 ± 0.07	3.68 ± 0.38	5.16 ± 0.22
P value	0.167	0.139	0.643	0.878	0.351	0.060	0.579	0.188	0.108	0.877
Day, 7										
C	177.14 ± 6.98	0.56 ± 0.42	4.09 ± 0.20	0.89 ± 0.04	0.69 ± 0.02	0.13 ± 0.07	0.06 ± 0.01	0.73 ± 0.07	9.45 ± 0.47	4.18 ± 0.13
IOPW	161.12 ± 7.80	0.62 ± 0.42	3.92 ± 0.20	0.90 ± 0.05	0.65 ± 0.02	0.30 ± 0.08	0.07 ± 0.01	0.62 ± 0.07	9.04 ± 0.53	4.39 ± 0.14
IOPW + L-car	165.11 ± 6.98	0.42 ± 0.49	3.95 ± 0.20	0.96 ± 0.04	0.72 ± 0.02	0.18 ± 0.08	0.08 ± 0.01	0.76 ± 0.07	10.27 ± 0.47	4.12 ± 0.13
P value	0.304	0.952	0.841	0.456	0.140	0.336	0.132	0.424	0.251	0.408

C Control, non—injection group, *IOPW* In ovo pure water injection group, *IOPW + L-car* In ovo L-carnitine injection group added pure water

Table 4 Morphological traits and relative asymmetry (RA) of bilateral morphological lengts of male broiler chicks exposed to in ovo L- carnitine injection

	Chick length	Body mass index	Head diameter	Beak length	Face laength	Middle toe length	Shank length	RA of bilateral lengts		
	cm	g/cm^2	mm	mm	mm	mm	mm	Face	Middle toe	Shank
Day, 0										
C	17.82 ± 0.13	0.15 ± 0.00	17.29 ± 0.32	6.40 ± 0.13^{a}	14.15 ± 0.20^{a}	16.00 ± 0.23^{a}	17.38 ± 0.24	1.47 ± 0.45	1.72 ± 0.05	1.58 ± 0.26^{a}
IOPW	17.94 ± 0.13	0.14 ± 0.00	16.46 ± 0.31	5.86 ± 0.12^{b}	13.03 ± 0.19^{b}	14.41 ± 0.21^{b}	17.00 ± 0.23	1.22 ± 0.42	2.33 ± 0.47	0.61 ± 0.25^{b}
IOPW + L-car	17.78 ± 0.13	0.15 ± 0.00	16.72 ± 0.31	5.94 ± 0.14^{b}	12.81 ± 0.19^{b}	13.72 ± 0.21^{c}	16.79 ± 0.22	1.48 ± 0.42	2.23 ± 0.47	0.86 ± 0.25^{ab}
P value	0.683	0.116	0.203	0.012	0.002	0.001	0.212	0.888	647	0.052
Day, 3										
C	22.38 ± 0.35^{a}	0.17 ± 0.01	18.72 ± 0.24	6.73 ± 0.09	13.79 ± 0.18	16.36 ± 0.30^{a}	20.22 ± 0.13	0.96 ± 0.03	0.76 ± 0.02	0.73 ± 0.02
IOPW	20.62 ± 0.35^{b}	0.17 ± 0.01	18.60 ± 0.34	6.33 ± 0.13	13.78 ± 0.25	15.18 ± 0.42^{b}	19.71 ± 0.18	1.01 ± 0.05	0.64 ± 0.03	0.82 ± 0.02
IOPW + L-car	21.20 ± 0.35^{b}	0.18 ± 0.01	18.88 ± 0.34	6.62 ± 0.13	14.24 ± 0.25	15.09 ± 0.42^{b}	20.02 ± 0.18	1.39 ± 0.04	0.98 ± 0.03	0.75 ± 0.02
P value	0.011	0.695	0.846	0.085	0.316	0.034	0.109	0.749	0.762	0.964
Day, 7										
C	26.36 ± 0.34	0.26 ± 0.01	20.25 ± 0.38	8.08 ± 0.15	15.19 ± 0.26	20.09 ± 0.29	23.54 ± 0.22	1.08 ± 0.03^{a}	0.68 ± 0.02	0.32 ± 0.02
IOPW	25.67 ± 0.38	0.24 ± 0.01	20.33 ± 0.42	8.27 ± 0.15	14.51 ± 0.26	19.62 ± 0.33	23.07 ± 0.22	0.83 ± 0.03^{b}	0.77 ± 0.02	0.47 ± 0.02
IOPW + L-car	26.10 ± 0.34	0.24 ± 0.01	19.78 ± 0.38	8.06 ± 0.15	15.31 ± 0.26	20.45 ± 0.29	23.54 ± 0.22	0.41 ± 0.03^{c}	0.59 ± 0.02	0.74 ± 0.02
P value	0.438	0.413	0.579	0.607	0.147	0.219	0.306	0.032	0.567	0.292

[abc] Different superscript means differ significantly $p < 0.05$

C Control, non—injection group, *IOPW* In ovo pure water injection group, *IOPW + L-car* In ovo L-carnitine injection group added pure water

membrane [13]. With respect to this statement, the developing embryo requires more energy provided by the breakdown of yolk membrane lipids in response to L-car at the internal pipping stage [18]. Therefore, in this study, lower TAS in the yolk membrane towards hatching than the initial TAS level of the egg yolk may be due to increased metabolic energy demand depending on the increasing age of broiler embryo as stated in a previous study [19]. L-carnitine activity in the liver has a main importance in response to the efficiency oxidation of fatty acid in the yolk membrane during the late embryonic development stage. In fact, IOL-car injection did not affect TAS in the liver tissue during the early posthatching developmental period. This finding indicates that the physiological requirements related to liver TAS of broiler chicks at this stage can be fulfilled by an adequate dose of L-car injected into the amnion membrane, and that can be met by sufficiently synthesized L-car in the liver. It has been reported that the requirement for L-car is increased during stressful periods with higher metabolic and physiological demands at the early development after hatching [20]. However, after hatching, liver TAS decreased linearly with increasing chick age, and male chicks injected with IOL-car had the lowest liver TAS compared to chicks of the control on day 3 of chick age. This indicated that hepatic non-enzymatic antioxidants were actively and efficiently involved in the antioxidant defence mechanism.

Plasma testosterone concentration was significantly affected by IOL-car admnistration and the highest concentration of plasma testosterone by effect of IOL-car was obtained at day 7 of chick age. This result was due to the interaction between IOL-car administration and the age of the chicks. Therefore, plasma testosterone can be increased by IOL-car administration during testicular development in male chicks at the posthatching stage, when testosterone is an essential hormone for the formation of the hypothalamic-pituitary–testicular axis, which determines secondary sexual characteristics and reproductive behaviour. L-car has been shown to increase the activity of reproductive hormones by improving the hypothalamic-pituitary–testicular axis [21]. Moreover, it has been found that the antioxidant influence of L-car on reproductive performance plays a role at the initial of sexual maturity [22]. The polyunsaturated fatty acids level in the cell membranes of the testes enhances during maturation, thereby increasing susceptibility to lipid peroxidation [23]. Therefore, L-car' antioxidant influence may be actively utilised from TAS in tissues due to increased activity of testosterone by improving and formation of hypothalamic-pituitary–testicular axis of broiler male chicks in this study. Furthermore, the resulting plasma testosterone concentration can be attributed to the increased follicle stimulating hormone in the blood of the male chicks because of the administration of L-car that causes high levels of total testosterone and follicle stimulating hormones as reported in resulted studies [24, 25].

It was reported that IOL-car applied at the different doses had no effect on posthatching development [26]. Similarly, the effect of IOL-car was found to be insignificant on organ development and chick weight during posthatching development in the present study. It was indicated that IOL-car injection into the air chamber of the embryonated egg did not affect on the utilisation of residual yolk membrane content at hatching, but residual yolk consumption increased with L-car dose at days 3 and 7 of chick age [27]. In this study, the injection of L-car into the amniotic membrane did not lead to this result at the same ages studied. The reason for the different effects of L-car on yolk membrane absorption in these two studies may be the application of L-car to different injection sites at day 18 of embryonic age. This highlights the importance of the choice of injection site [28] and the fact that L-car at the different injection sites affect utilisation of residual yolk membrane with different physiological effects at an early stage of chick development. The body length and the middle toe length of male chicks reduced by the effect of IOL-car at day 3 of chick age. The beak, face and middle toe lengths of newly hatched chicks reduced in the IOL-car group. The RA of bilateral traits were unchanged with IOL-car administration, but the RA of face length in the IOPWL-car group was found to be lower than control group at day 7 of chick age. All these significant results showed that the developmental parameters revealed age-specific effects.

Conclusions

In conclusion, L-carnitine affects chick' morphological traits and total antioxidant levels in the tissues as a function of age, and increases testosterone level interacted with the formation of the hypothalamic-pituitary–testicular axis of male broiler chicks at an early posthatching developmental period.

Abbreviations

L-car	L-carnitine
TAS	Total antioxidant status
IO	In ovo
PW	Pure water
RA	Relative asymmetry
ELISA	Enzyme-linked immunosorbent assay
KCL	Potassium chloride

Acknowledgements

The study was conducted in The Research and Application Farm Hatchery of Van Yuzuncu Yil University. The Research and Application Farm Hatchery of Van Yuzuncu Yil University is gratefully acknowledged.

Authors' contributions
The author of this study is Elif BABACANOĞLU ÇAKIR and it has only an author. No other author contributions.

Funding
The study is not funded.

Data availability
Data from this study are available from the author on reasonable request.

Declarations

Ethics approval and consent to participate
The author confirms that the journal's ethical policies have been followed and that EU standards for the protection of animals used for scientific purposes have been met. The study was approved by the Committee for the Ethical Care and Use of Animals of the Van Yuzuncu Yil University (Approval number: 2023/01–02).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 25 November 2024 Accepted: 14 March 2025
Published online: 27 March 2025

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