Original Article

Effect of in Ovo L-Carnosine Injections on The Oxidative Status and Physiological Homeostasis of Broiler Chicks

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Abstract

The study investigated the effect of *in ovo* L-carnosine injections on the alteration of homeostatic imbalance in newly hatched broiler chicks. A total of 400 fertilized eggs were allocated into four experimental groups, namely a control (LC0) with no injection, a sham group receiving normal saline (INS), and two treatment groups receiving 30 µg (IC30) and 45 µg (IC45) of L-carnosine, respectively. The injections were administered on the 18th day of incubation. Key parameters assessed included hatchability (EH) and egg pecking percentages (EP), initial weight of hatched chicks (IW), and weight at seven days post-hatching (W7). Blood samples were collected from the jugular vein to examine a range of vital indicators. Results showed that the *in ovo* L-carnosine injection had no significant effects on EH and EP, the activity of the enzymes aspartate transaminase (AST), alanine aminotransferase (ALT), alkaline phosphatases (ALP), catalase (CAT), and concentrations of malondialdehyde (MDA) and glutathione (GSH). A significant decrease was noted in peroxide value (PV) at IC30 and free fatty acid (FFA) at IC45 while superoxide dismutase (SOD) activity at IC30 and glutathione peroxidase (GPX) activity at IC45 increased markedly. There was a significant decrease in the concentration of corticosterone (CORT) at IC30 and IC45 and a significant increase in the concentration of leptin (LP) at IC30 and IC45 compared to LC0. A significant decrease in IW at IC45 occurred compared to LC0, which did not persist until the seventh day of age, as the differences at W7 became insignificant. Overall, the findings suggest that *in ovo* injections of L-carnosine may influence certain hemostasis indices in newly hatched chicks, although its effects on hatchability and growth parameters require further investigation.

Keywords: Dipeptides, Oxidative stability, ROS, hatching

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Introduction

Hatching is the initial production cycle in chicken meat production. Billions of hatched chicks are produced worldwide annually under stressful conditions (11). The chicks are subsequently exposed to stress during transport from the hatchery to the rearing house, as evidenced by (31) who noted an increase in chick mortality rates during the process. Stress is typically indicated by elevated CORT, which is associated with a decrease in chick vitality and growth problems, with high CORT leading to an increase in respiration rate in stressed embryos (13). Hanafi et al. (10) observed an increase in CORT on the 14th day of incubation, followed by a decrease on the 18th day, and a subsequent increase after hatching, because of stress during hatching and transportation. The effects of exogenous CORT result in a reduction in embryonic fiber area ratio, which is reflected in subsequent muscle growth (3), as well as disruption of mitochondrial biosynthesis and dysfunction and perturbation of energy metabolism (19). CORT contributes to the regulation of white fat metabolism gene expression in chicken muscle (12). Kadhim and Kuenzel (15) observed a clear concordance in the expression pattern of the TSHβ gene of the hypothalamus-pituitary-thyroid axis with increased corticosterone levels in broiler chickens, that also leads to the inhibition of numerous anabolic hormones.

Consequently, it is important to identify solutions for this stress during the incubation period, as it is related to post-hatching growth, given that incubation and hatching conditions affect the quality and welfare of the embryo and thus the post-hatching growth trajectory **(28)**. Research efforts should focus on the hatching stage, which accounts for 33-38% of the bird's life **(5)**. Mierzejewska **(20)** indicated that high concentrations of L-carnosine in the muscles of broilers reduce oxidative stress and improve growth indicators when its concentration in muscles increases **(17)**.

L-carnosine is a dipeptide molecule synthesized in animal tissues when the basic amino acid β-alanine reacts with the basic amino acid L-histidine, which is why it is also called β-alanylhistidine, and its chemical formula is $C_9H_{14}N_4O_3$ **(6)**. It exhibits significant activity in regulating muscle pH, which ranges from 6.4 - 7.4, which in neutral or near-neutral, and confers a key role in regulating cellular pH, particularly under acidic conditions during stress **(14)**. It represents an effective strategy to reduce oxidative stress by inhibiting free radicals **(25)**, regulating hydroxyl radicals, activating SOD **(32)**, and playing a crucial role in metal chelation **(18)**. Furthermore, research has demonstrated that *in ovo* injection of 0, 25 and 50 µg L-carnosine on the 7th and 18th day of incubation resulted in a significant increase in the mean weight of hatched chicks and relative body stability when a concentration of 50 µg was administered on the 18th day of embryonic development **(16)**.

This study investigates the role of L-carnosine in the oxidative stability of newly hatched chicks and its effects on hatchability, chick vigor, and a range of relative homeostasis indices.

Materials and Methods

Four hundred fertilized eggs of Ross 308 chickens averaging 60.49 (g)/egg were incubated in one of the hatcheries of the Shukr hatchery. During their transfer to the hatchery, a candling was performed to identify unfertilized eggs and dead embryos. Subsequently, 400 fertilized eggs were selected and divided equally into four groups (one hundred/group), comprising the control group without injection (LC0), sham group with normal saline injection (INS), and *in ovo* injections of 30 µg (IC30) and 45 µg (IC45).

Preparation of the Injection Solutions

Normal saline solution

The normal saline solution was prepared 24 hours before injection by adding 8.5 g NaCl (99.9% purity) to a volumetric flask. Distilled water was then added to the glass flask in batches to raise the volume to 1000 ml. The solution was placed on a magnetic stirrer until the NaCl had completely dissolved and the solution was clear and free of sediment. The pH was adjusted and confirmed at 5.5. The solution was then sterilized in an autoclave for 15 minutes, then L-carnosine weights were added, and the solution was stored in the refrigerator until use.

L-carnosine solution at 30 and 45 micrograms/egg

L-carnosine from Belle Chemical LLC was used at 100% purity, and the concentrations were converted from 30 and 45 micrograms to 0.03 and 0.045 milligrams, respectively.

Dilution was calculated using the standard equation $C_1V_1 = C_2V_2$, where

C_1: Original concentration (before dilution)

V_1: Original volume of the solution (before dilution)

C_2: Final concentration (after dilution)

V_2: Final volume of the solution (after dilution)

Then the L-carnosine solution was prepared and the hydrogen ion concentration measured (7.1). The solution was then stored in the refrigerator until use.

In ovo injections

The *in ovo* injections were performed on the 18th day of incubation using an automatic syringe with a 22G needle of 13 mm length at a dose of 0.1 ml/egg. A hole was then drilled in the wide end of egg using an electric drill. The hole was then sealed with paraffin wax and the eggs transferred to the incubator maintained at a temperature of 37 °C and a humidity of 75%. The shell area above the injection site was sterilized before and after drilling.

Hatchability (EH) and egg pecking (EP) percentages

At the end of hatching, the number of hatched chicks from each group was recorded. The unhatched eggs were then broken to count the number of dead chicks in the shell. The percentage of each group was calculated by dividing the partial number (hatched chicks or unhatched eggs) by the total number and multiplied by one hundred.

Body weight

Body weight was taken immediately after hatching (IW) and on 7th day post-hatching (W7). The weights of the chicks were determined individually to two decimal places using sensitive scales. The difference in weight on day seven compared to the time of hatching was then calculated to determine weight gain (GW).

Blood sample collection

Immediately after hatching, blood was collected from the jugular vein of 10 birds per replicate using a 1 ml syringe and a 25 G needle. The blood was then drained into a gel tube, centrifuged at 10,000 rpm for 10 minutes, and the serum transferred to an Eppendorf tube. The tube was placed in a cool box with ice and transported to the laboratory to perform the following tests.

Laboratory Assays

The steps outlined in the protocol included with each kit were applied, as follows:

AST assay

Serum at 5 µl from each sample was set up into the wells of a microplate, and 50 µl of the AST reagent solution was added to each well. The wells were sealed using adhesive film and incubated at 37 °C for 10 minutes. The adhesive film was carefully removed and 50 µl of the AST color reagent was introduced into the wells. The adhesive film was applied to the wells, and the plate was incubated at 37 °C for 10 minutes. Subsequently, the adhesive film was removed and 200 µl of 0.1 M HCl were introduced into the wells, and the absorbance was measured at a wavelength of 510 nm. The activity of the AST enzyme was determined by mapping the reading onto a standard curve that included the average absorbance of each oxaloacetate standard across successive dilutions.

ALT assay

Serum at 10 µl from each sample was added to the microplate wells, following which 50 µl of ALT reagent solution was added to each well. Adhesive film was attached to the wells and incubated at 37 °C for 10 minutes. Then, 50 µl of DNPH color solution was added to the wells after the adhesive covering was carefully removed. The adhesive film was placed on the wells and the plate was incubated at 37 °C for 10 minutes. The adhesive sheet was removed and 200 µl NaOH (0.5 M) was added to the wells. Absorbance was checked at 510 nm. The measurement was projected onto a standard curve of the average absorbance of each oxaloacetate standard for subsequent dilutions to quantify ALT enzyme activity.

ALP assay

Diethanolamine buffer at 200 µL (DEA, 6.25 mM, pH 9.8) containing 1 mM $MgCl_2$, 40 µL APP (800 mM), 20 µL ALP activities at various concentrations, and 240 µL tris-glycine buffer (10 mM, pH 9.5) were each added to an Eppendorf tube and the solution was incubated at 37 °C for 45 min. After the reaction was complete, 40 µL of 0.75 M diethylenetriamine (DETA) was added to the above solution and mixed well. The resulting solution was placed in a water bath at 60 °C for 40 minutes. The fluorescence emission spectrum data was recorded followed by an excitation wavelength of 376 nm. The absorption spectrum was recorded from 300 to 600 nm.

MDA assay

BHT at 10 µl was added to a tube, followed by 250 µl each of serum, acid reagent, and TBA reagent. The tube was returned after capping and shaken rapidly five times. It was then incubated at 60 °C for 60 minutes and then centrifuged at 10,000 rpm for 2-3 minutes. The reaction mixture was then placed in a cuvette and the wavelengths between 300-700 nm recorded.

PV assay

Deionized water at 50 µl was added to each well of the microplate in duplicate. Standard hydrogen peroxide at 50 µl was added to each well of the microplate and 50 µl of the diluted serum was added to each well. Colorimetric hydrogen peroxide indicator at 100 µl was added to each well of the microplate. Mixing was performed by gently tapping the side of the microplate for 10 seconds. The microplate was incubated at room temperature for 30 minutes. The optical density (OD) of each well was measured using a microplate reader at a wavelength of 550 nm.

FFA assay

Serum at 10 µl was added to the wells of a microtiter plate, then 200 µl of the 1X enzyme mixture A was added. The walls were covered with an opaque cover to protect them from light. The microtiter plate was incubated at 37 °C for 30 minutes, then left at 4 °C, and 100 µl of the detection enzyme mixture added. The walls were covered with an opaque cover to protect them from light and incubated at 37 °C for 10 minutes. The data was read using a fluorescence microplate reader equipped with a range of 530 – 560 nm for excitation

and 585 – 595 nm for emission. The concentrations of free fatty acids were calculated by comparing the sample values with the standard curve.

ELISA assay

ELISA assay was used to assess the activity of the enzymes CAT, GSH and GPX as well as the concentration of the hormones CORT and LP with the following kits:

1) My bio source products

- Chicken corticosterone ELISA kit (CORT Elisa kit)
- Chicken leptin ELISA kit (LEP Elisa kit)
- Catalase (CAT) ELISA kit for chickens (CAT Elisa kit)
- Chicken superoxide dismutase (SOD) ELISA kit (SOD Elisa kit)

2) E-lab science products

- Chicken glutathione ELISA kit (GSH Elisa kit)
- Glutathione peroxidase (GSH-Px) activity assay kit (E-BC-K096-M)

Statistical Analysis

The study data was analyzed using GraphPad Prism software, which included a one-way analysis of variance and Tukey's HSD test for comparing significant differences between means.

Results and Discussion

There were no significant differences in EH and EP among the four experimental groups (Table 1).

Table 1. Effect of L-carnosine *in ovo* injections on hatchability and egg pecking percentages

	Percentage	
Treatments	Hatchability	Egg pecking
LC0	94.00 ± 2.39	6.00 ± 2.39
INS	94.00 ± 2.39	6.00 ± 2.39
IC30	96.00 ± 1.97	4.00 ± 1.97
IC45	95.00 ± 2.19	5.00 ± 2.19
Sig.	NS	NS

LC0: control without injection; INS: *in ovo* injection of 0.1 ml normal saline; IC30: *in ovo* injection of 30 µg L-carnosine; IC45: *in ovo* injection of 45 µg L-carnosine. NS: not significant.

It was found that the highest IW was recorded in LC0 with significant differences compared to INS and IC30. There were no significant differences in W7 and GW among the four experimental groups (Figure 1).

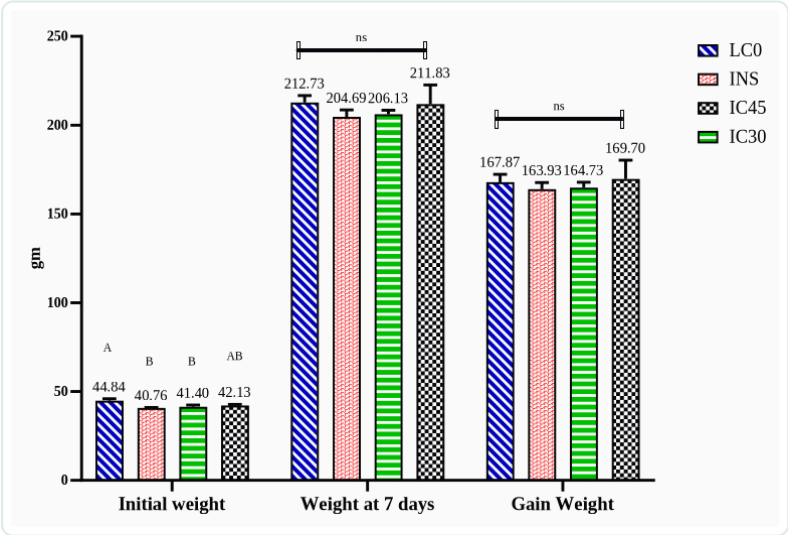

Figure 1. Effect of *in ovo* L-carnosine injections on initial weight (IW), body weight 7 days post-hatching (W7) and gain weight (GW)

The results showed that *in ovo* injection with L-carnosine did not lead to any significant differences in the activity of the enzymes AST, ALT, ALP, and the MDA concentration compared to LC0 and INS (**Tables 2** and **3**).

Table 2. Effect of *in ovo* injections with L-carnosine on AST, ALT, and ALP activity (U/L)

	Enzyme activity (U/L)		
Treatments	**AST**	**ALT**	**ALP**
LC0	8.34 ± 3.31	5.14 ± 1.21	5.50 ± 1.83
INS	3.06 ± 0.74	7.37 ± 3.12	2.14 ± 1.22
IC30	6.01 ± 4.85	18.81 ± 9.63	4.28 ± 2.14
IC45	9.15 ± 2.53	19.81 ± 13.37	12.83 ± 5.72
Sig.	NS	NS	NS

LC0: control without injection; INS: *in ovo* injection of 0.1 ml normal saline; IC30: *in ovo* injection of 30 µg L-carnosine; IC45: *in ovo* injection of 45 µg L-carnosine. NS: not significant. AST: aspartate transaminase; ALT: alanine aminotransferase; ALP: alkaline phosphatase.

Table 3 shows a significant decrease in PV for IC30 compared to LC0, while IC30 showed no significant differences compared to INS. A notable decrease in FFA was observed at IC45 compared to INS, while there was no significant differences between IC45 and IC30 compared to LC0.

Table 3. Effect of *in ovo* injection with L-carnosine on non-enzymatic antioxidants

	Non-enzymatic antioxidants		
Treatments	**MDA (mmol/L)**	**PV (mmol/L)**	**FFA (µM)**
LC0	0.95 ± 0.11	0.74 ± 0.05^{a}	1.65 ± 0.12^{ab}
INS	0.77 ± 0.03	0.59 ± 0.02^{ab}	1.97 ± 0.14^{a}
IC30	0.81 ± 0.05	0.45 ± 0.04^{b}	1.46 ± 0.12^{ab}
IC45	0.81 ± 0.05	0.78 ± 0.04^{a}	1.25 ± 0.07^{b}
Sig.	NS	0.05	0.05

LC0: control without injection; INS: *in ovo* injection of 0.1 ml normal saline; IC30: *in ovo* injection of 30 µg L-carnosine; IC45: *in ovo* injection of 45 µg L-carnosine. Different letters on columns indicate significant differences among the mean values at the 0.05 probability level NS: not significant. MDA: malondialdehyde; PV: peroxide value: FFA: free fatty acids.

The activity of the CAT enzyme showed no significant differences between the experimental groups for the L-carnosine *in ovo* injections (**Table 4**). There was a significant increase in SOD at IC30 compared to LC0, whereas no significant differences were observed with INS. IC45 also showed no significant differences in SOD compared to LC0 and INS.

Table 4. Effect of *in ovo* injection with L-carnosine on catalase and superoxide dismutase activity

Treatments	**CAT (U/minute/mL)**	**SOD (U/mL)**
LC0	1.71 ± 0.07	178.92 ± 15.50^{b}
INS	1.74 ± 0.14	341.67 ± 9.09^{ab}
IC30	1.22 ± 0.21	454.17 ± 40.56^{a}
IC45	1.62 ± 0.21	278.67 ± 70.95^{ab}
Sig.	NS	0.05

LC0: control without injection; INS: *in ovo* injection of 0.1 ml normal saline; IC30: *in ovo* injection of 30 µg L-carnosine; IC45: *in ovo* injection of 45 µg L-carnosine. Differences among the mean values at the 0.05 probability level. NS: not significant. CAT: catalase; SDD: superoxide dismutase.

Table 5 shows a significant increase in GPX activity for IC45 compared to LC0 and INS, while no significant differences were observed between IC30 and LC0, with an increase at IC30 compared to INS, which was not significantly different from LC0. *In ovo* injection of L-carnosine showed no significant differences in GSH enzyme activity in the four experimental groups.

Table 5. Effect of *in ovo* injection with L-carnosine on glutathione and glutathione peroxidase

Treatments	GSH (mmol/L)	GPX (µmol/min/ml)
LC0	10.78 ± 0.85	8.77 ± 1.16^{bc}
INS	9.22 ± 1.20	6.92 ± 0.53^{c}
IC30	11.67 ± 1.17	10.36 ± 0.80^{ab}
IC45	6.94 ± 1.80	11.91 ± 1.21^{a}
Sig.	NS	0.05

LC0: control without injection; INS: *in ovo* injection of 0.1 ml normal saline; IC30: *in ovo* injection of 30 µg L-carnosine; IC45: *in ovo* injection of 45 µg L-carnosine. Differences among the mean values at the 0.05 probability level. NS: not significant. GSH: glutathione; GPX: glutathione peroxidase.

CORT decreased significantly for IC45 compared to LC0 and INS. It decreased significantly in IC30 compared to LC0 and computationally compared to INS. No significant differences were found between IC45 and IC30 (**Figure 2**).

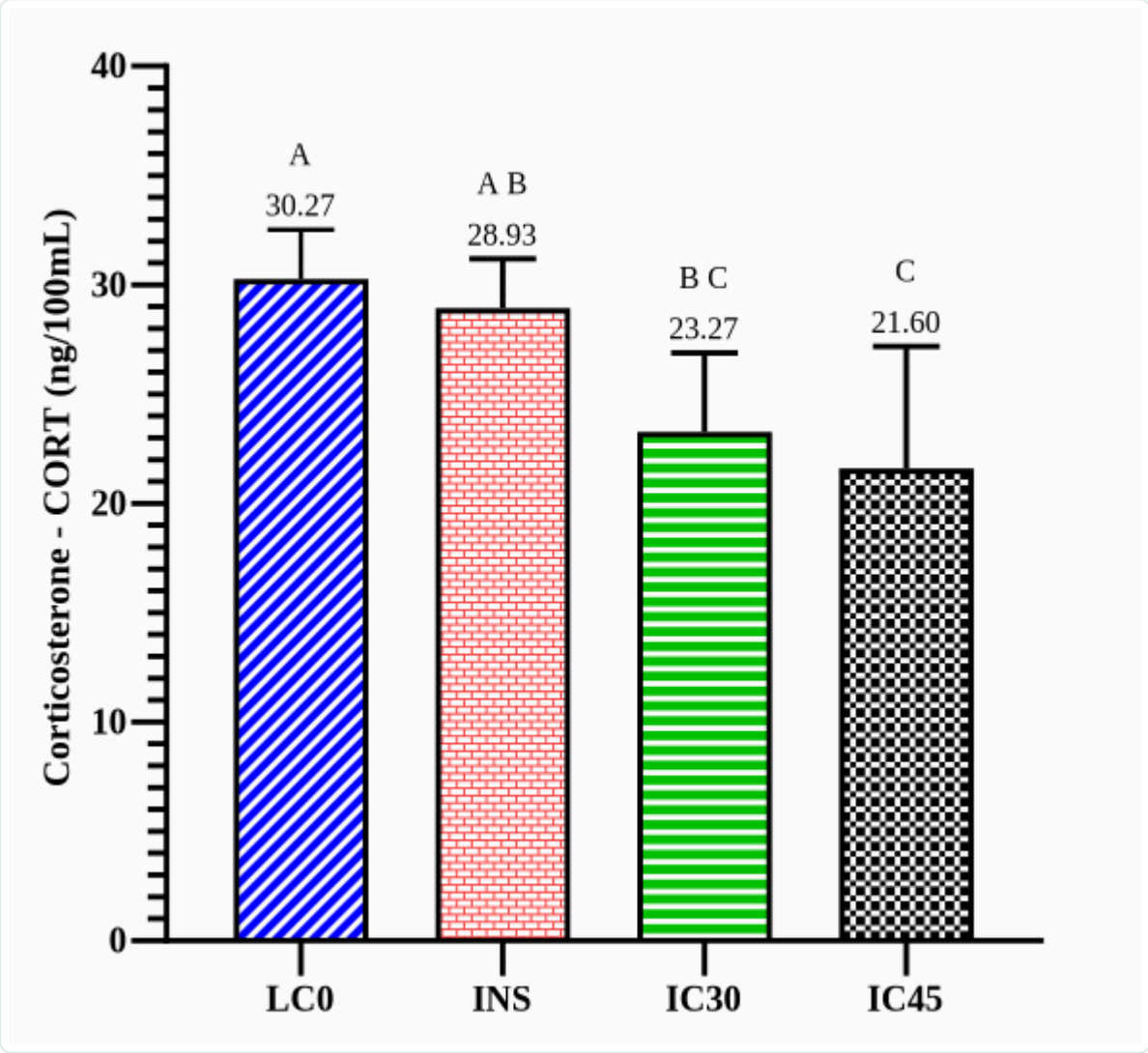

Figure 2. Effect of *in ovo* injection with L-carnosine on corticosterone concentrations - CORT (ng/100mL)

LP significantly increased for IC30 and IC45 compared to LC0, while no notable differences were found between the three groups compared to INS (**Figure 3**).

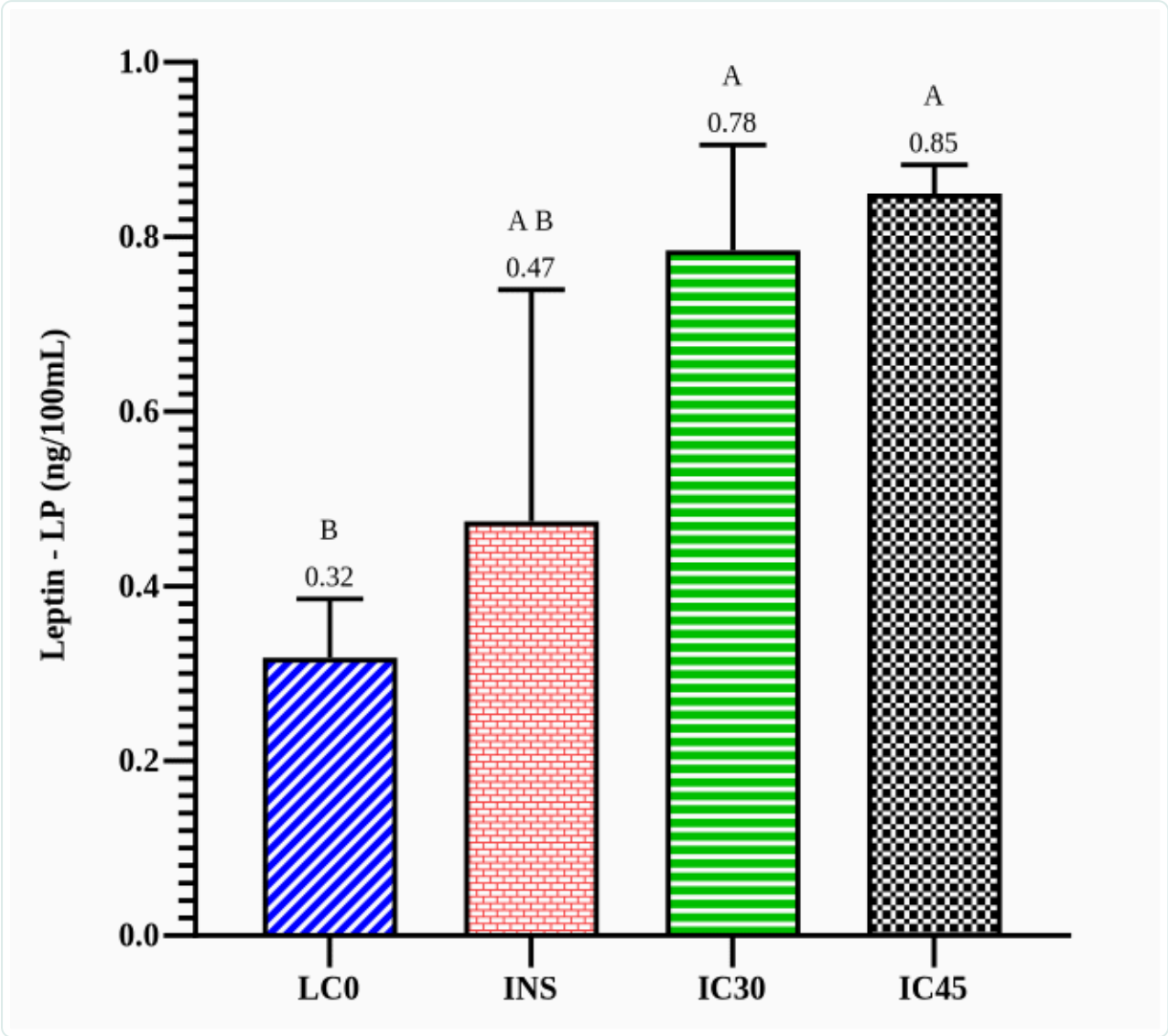

Figure 3. Effect of *in ovo* injection with L-carnosine on leptin concentrations - LP (ng/100mL)

No significant differences were observed in EH and EP rates, indicating the safety and precision of the injection procedure, as improper implementation can result in embryonic mortality or weakening **(23)**.

The notable decrease in IW may be attributed to individual embryonic variations or other factors such as impartiality in hatching eggs selection and optimal incubation conditions. The significant differences were no longer apparent when W7 was recorded (**Figure 1**), potentially signifying a state of homeostasis resulting from oxidative balance under the influence of L-carnosine at IC30 and IC45, as noted by Antwi-Boasiako et al. **(4)** that carnosine-derived antioxidants inhibit free radicals. This study showed a significant reduction in FFA at IC45 (**Table 3**) and PV at IC30, contrasting with an increase in SOD activity (**Table 4**) and GPX enzymes (**Table 5**). This suggests a harmonization of non-enzymatic and enzymatic antioxidants in mitigating ROS and their deleterious effects, maintaining oxidative stability in cells, thereby protecting unsaturated fatty acids, nucleic acids, proteins, etc. This preservation of relative stability allows for optimal growth in the bird **(22)**, as further reflected in the W7 measurements (**Figure 1**).

SOD catalyzes the conversion of superoxide (O_2^-) to hydrogen peroxide (H_2O_2) while GPX neutralizes it to water and molecular oxygen, resulting in decreased ROS levels (2). This process is accompanied by a reduction in ROS-producing enzyme activity, followed by the release of iron and copper from the protein and the maintenance of mitochondrial homeostasis (8). L-carnosine may be involved due to its chelating properties for copper, manganese, zinc, and iron ions (1). It may be essential for SOD synthesis, as it has been shown to transport copper, zinc, and manganese ions into cells or specifically into mitochondria. Furthermore, it inhibits the oxidation of DCF-DA by H_2O_2 in the mitochondria, serving protective role against free radicals, with evidence of oxidative roles played by L-carnosine in the activation of the various reactions in mice (9).

However, no adverse effects on cellular functions were observed following L-carnosine injection, as evidenced by the absence of significant differences in AST and ALT (Table 2). This elucidates the state of harmony between the non-enzymatic and enzymatic antioxidant systems and the stability of the redox state. It also supports the involvement of L-carnosine in stimulating Q10 production, such that one complements the role of the other in enhancing mitochondrial functions and preventing oxidative stress in mice (26), suggesting a similar role in broiler chickens. In this study, a significant increase in GPX activity was found (Table 5), as characterized by its ability to recycle, especially when accompanied by an increase in GSH activity (24), and this is what the results of this study achieved (Table 5).

One of the important roles of L-carnosine is to contribute to regulating muscle pH towards neutrality, especially under stress conditions, as it acts as a proton carrier to accelerate the release of hydrogen ions into the cytoplasm, which contributes to pH stability (30). Low concentrations of L-carnosine in muscles cause various problems, especially pectoral muscle diseases (27). In addition, it has antioxidant and chelating properties that ensure a balanced internal environment of the cells, as evidenced by the low concentration of the hormone CORT in blood serum (Figure 2). Elevated levels of CORT in muscles disrupt and delay metabolic processes, which affects the cell cycle and consequently delays cell growth and development (7). This could explain the results of (Figure 1), as there were no significant differences between the experimental treatments in W7 after IC30 recorded a significant decrease in IW, and with non-significant differences in GW (0-7 days). However, the lowest average was recorded at LC0 to match the increase in CORT (Figure 2).

The significant increase in LP (Figure 3) is another indicator of the role of L-carnosine in maintaining relative stability, as it regulates energy metabolism and its balance, cell growth, and development by stimulating gene expression of a number of neuropeptides in the hypothalamus (29). Also, disturbances in LP levels under the influence of various environmental conditions lead to various metabolic diseases (21). Energy balance and metabolism are one of the most prominent concepts for oxidative balance in the body and, thus, for homeostasis, and this is what the results of this study have achieved.

Conclusion

The *in ovo* injection of carnosine before hatching modifies the response of stressed chicks and improves their activity in restoring relative physical stability by regulating the oxidation and reduction systems in cells. Further studies can aid in verifying the effect of carnosine injection on hatching characteristics.

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Author Contributions: Both authors have read and agreed to the published version of the manuscript.

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مقالة أصيلة

تأثير حقن بيض التفقيس بالكارنوزين في عدد من مؤشرات الحالة التأكسدية والأستتباب البدني النسبي لأفراخ فروج اللحم

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الخلاصة

أن الهدف من هذه الدراسة بيان تأثير حقن البيض بالكارنوزين في تعديل اختلال الاتزان البدني النسبي لدى أفراخ فروج اللحم الفاقسة حديثاً. لذلك خصصت 400 بيضة مخصبة قسمت على أربعة مجموعات تضمنت الأتي: LC_0: مجموعة سيطرة من غير حقن؛ INS: حقن البيض بالمحلول الملحي الفسيولوجي؛ IC_{30}: حقن البيض 30 مايكروغرام كارنوزين؛ IC_{45}: حقن البيض 45 مايكروغرام كارنوزين. أجريت عملية الحقن عند اليوم 18 من الحضانة. ثم حسبت نسب البيض الفاقس والبيض الكابس، والوزن الابتدائي والوزن عند 7 يوماً، ثم سحب الدم من الوريد الوداجي لدراسة عدد من المؤشرات الحيوية. وقد بينت النتائج ان حقن البيض بالكارنوزين لم يؤثر معنوياً في نسب البيض الفاقس والكابس ونشاط إنزيمات Aspartate transaminase (AST) و Alanine aminotransferase (ALT) و Alkaline phosphatases (ALP) و Catalase (CAT) وتركيز Malondialdehyde (MDA) و Glutathione (GSH). وانخفاض معنوي في Peroxide value (PV) للمعاملة IC_{30} والأحماض الدهنية الحرة (FFA) للمعاملة IC_{45}. وارتفاع معنوي في نشاط إنزيم Superoxide dismutase (SOD) للمعاملة IC_{30} ونشاط Glutathione peroxidase (GPX) للمعاملة IC_{45}. وانخفاض معنوي في تركيز Corticosterone (CORT) لدى IC_{30} و IC_{45}، وارتفاع معنوي لدى IC_{30} و IC_{45} في تركيز Leptin (LP) مقارنةً مع LC_{30}. مع انخفاض معنوي في معدل الوزن الابتدائي للأفراخ الفاقسة للمعاملة IC_{45} مقارنةً مع LC_0، الذي لم يستمر إلى اليوم السابع من العمر إذ أصبحت الفروق غير معنوية في معدل وزن الجسم عند 7 يوم بعد الفقس. بشكل عام، تشير النتائج إلى أن حقن الكارنوزين في البيضة قد يؤثر في بعض مؤشرات الثبات البدني النسبي لدى الأفراخ الفاقسة، مع ذلك، أن تأثير الكارنوزين في قابلية الفقس ومعايير النمو يتطلب دراسات أخرى

الكلمات المفتاحية: البيتيدات الثنائية، الثبات التأكسدي، التفقيس