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IMPACT OF *IN OVO* INJECTION WITH SPIRULINA IN MITIGATING NEGATIVE EFFECTS OF THERMAL MANIPULATION DURING EMBRYOGENESIS OF SILVER SABAHIA CHICKEN STRAIN

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ABSTRACT: This study was proposed to highlight the impact of *in-ovo* injection with *Spirulina platensis* (SP) solutions in alleviating the negative effect of thermal manipulation during incubation. Also, estimate the influence of this treatment on embryonic development, hatching traits, physiological performance and the expression of gene HSP70. A total of 750 hatching eggs from a 45- wk-old of Sliver Sabahia chickens strain were numbered and weighed (51.00 ± 0.6 g). All the eggs incubated at 37.5°C (99.5 F°) and 55% relative humidity (RH) from 1 d to 11 d of incubation , then were subjected to daily thermal manipulation at 39.5°C (103.1 F°) and RH (60%) for four hours from 12th to 18th day of incubation. At 14th day of incubation, 660 fertilized eggs with evidence of living embryos were randomly assigned to four groups of 165 eggs each (3 replicates of 55 eggs each). The 1stgroup: Eggs without injection, served as (control group), The 2nd group: Eggs injected with saline solution, served as sham control (1 ml saline solution /egg), The 3rd group: Eggs injected with 0.2 mg SP /egg, the 4th group: Eggs injected with 0.4 mg SP /egg. Results conducted that Injecting eggs with SP at different concentrations (0.2 or 0.4 mg/egg) decreased egg weight loss (%) , yolk sac (%), embryonic mortality ($P \leq 0.01$), pipped eggs (%) and embryonic malposition ($P \leq 0.01$) compared to control groups. While, significantly ($P \leq 0.01$) improved embryonic development, hatchability percentage, chick weight and quality, most of blood parameters and the expression of gene HSP70 as compared to control groups. The best results were obtained by *in ovo* feeding of SP at 0.4 mg /egg.

In conclusion, *In ovo* feeding *Spirulina platensis* solution with 0.4 mg/egg during thermal manipulation created new hormonal, immune systems and increasing levels of HSP70 in the embryos with more ability to withstand and challenge the temperature increase without any deleterious effect, this was reflected in improvement of hatching traits and chick quality.

Key words: Spirulina, *in ovo* injection, thermal manipulation, hatchability,gene expression HSP70

INTRODUCTION

Thermal manipulation during embryogenesis is considered as an applicable tool for growing chicks to cope with thermal stress during the summer months and especially at areas which suffering from temperature increase (Khalil *et al*., 2024). While, the deleterious effect of exposing embryos to high temperature through incubation may cause embryonic malposition and dysfunction in many vital organs such as heart, liver, and endocrine glands (Buckiova *et al*.,2003), early hatching (Leksrisompong *et al*.,2007), slower embryonic development , reduced carbohydrate and lipid metabolism (Willemsen *et al*., 2010) and diminished chick quality (Piestun *et al*., 2009).

In commercial poultry rearing, the technique of injecting eggs with antioxidant substances in organic form is considered one of the best methods to ensure the complete and safe delivery of nutrients to the embryo, lower the stress and embryonic mortality during the late phase of incubation (Hanaa *et al*., 2023), in addition to support their immunity system (El-Saadany *et al*., 2019). Consequently, improving the hatching rate and increasing the weight of chicks by modifying the morphology of the fetal intestine (Rufino *et al*., 2019).

Spirulina platensis (SP) is considered one of the richest algae in nutrients, as dried *Spirulina* powder contains approximately 70% protein, 20% carbohydrates, 7% lipids, fatty acids, vitamins, and minerals (Brito *et al.*, 2020; Mishra *et al.*, 2023). In addition to the nutritional enrichments, *Spirulina* contains different antioxidant phytochemicals like phycocyanin, phycobiliproteins, allophycocyanin with the ability of radical scavenging and improve the antioxidant defense system (Hayashi *et al*., 2009). Also, it contains pigments such as ß-carotene and zeaxanthin, provides promising biological activity as an antioxidant, anti-inflammatory, antibacterial, antineoplastic and immune booster (Agustini *et al.*, 2015; Farag *et al.*, 2016; Chaudhary *et al.*, 2023).

Hajati *et al*. (2021) found that feeding the embryos with SP before hatching reduced yolk residue and serum malondialdehyde, improved hatchability, antioxidant status and weight of Japanese quails at hatch. In addition, Mirzaie *et al*. (2018) reported that dietary SP supported the immune system of broiler chickens exposed to heat stress condition. Moreover, Swanson *et al*. (2012) demonstrated that fatty acids in SP could be promote the growth of embryos and prevent being sick.

The most common heat shock protein is HSP70, which protects organisms from heat toxic effects, and its expression is responsible for an endogenous mechanism of stress adaptation in living cells. (Hao, *et al.*, 2012; Gu, *et al.*, 2012). Sharp decrease caused by acute heat stress in the total cell protein synthesis of chickens. When HSPs synthesis continues to increase, the total protein content of cells gradually increases, suggesting that HSPs have protective and reparative effects on cells in a high-temperature environment (Xie, *et al.*, 2018). On the other hand, Hajati *et al.* (2020) reported that under heat stress, there was no difference in gene expression of HSP70 in the liver of quails fed diet supplement with SP.

Accordingly, the aim of the current manuscript was investigating the dual purposes of injection *Spirulina* solutions in eggs of Silver Sabahia chickens strain coincided with thermal manipulation during incubation on embryogenesis, hatching traits, chick quality, blood parameters and immunity-related genes expression

MATERIAL AND METHODS

The current trial was conducted at El–Sabahia Poultry Research Station, Animal Production Research Institute,

Agricultural Research Center, Ministry of Agriculture, Egypt.

Preparation of *Spirulina platensis* solutions

Spirulina platensis powder (SP) was purchased from a private Egyptian Company (Imtenan Company at San Stefano, Alexandria, Egypt). A mass 33 mg of SP powder was dissolved in 165 ml of saline solution (0.9 Nacl) to obtain solution of 0.2 mg SP/ml saline. As well, it was dissolved 66 mg of SP powder in 165 ml of saline to obtain solution of 0.4 mg SP / ml saline. Then, the solutions kept in refrigerator at 4°C until used.

Egg injection method

Before injecting the eggs, the injection site was cleaned with 70% ethanol alcohol, and then eggs were punctured with a thin and hard stylus. SP solutions was injected by using hypodermic needle (25mm long) in yolk sac by using a flashlight to find its location. Immediately after the egg injected, the hole was covered with wax using a wax gun and eggs were returned to the incubator. Solutions of treatments were warmed to 30°C before injection.

Experimental design

A total of 750 hatching eggs from a 45-wk-old of Sliver Sabahia chickens strain (Aly *et al*., 2017) was numbered and weighed (51.00 ± 0.6g). All eggs were incubated at 37.5°C (99.5 F°) and 55% relative humidity (RH) from 1 day until 11 days of incubation , then all eggs were subjected to daily thermal manipulation at 39.5°C (103.1 F°) and RH (60%) for four hours from 12^{th} to 18^{th}daysof incubation . All egg trays were set and distributed randomly at different places inside the incubator to reduce the effect of tray position on embryos growth. On the 14^{th} day of incubation, 660 fertilized eggs with evidence of living embryos were randomly assigned to four groups of 165 eggs each (3 replicates of 55 eggs in each). These groups were treated as follows:

The 1^{st} group: Eggs without injection, served as (control group),

The 2^{nd} group: Eggs injected with saline solution (0.9 Nacl), served as sham control (1 ml saline solution /egg)

The 3^{rd}group: Eggs injected with 0.2 mg SP /egg

The 4^{th}group: Eggs injected with 0.4 mg SP /egg

For each group, the injection volume was standard (1 ml per egg). On d 18 of incubation, the eggs were transferred singly into pedigree hatching baskets and then placed into hatcher for the remainder of the incubation period at 37.2°C (98.6°F) and 70% RH for 3 days till the hatch.

Incubation event and chick quality assessment

All eggs were weighed individually again during incubation on the 12^{th} and 18^{th} day in order to obtain egg weight loss percentages for each incubation time. At days 12 and 18 during incubation, forty eight eggs were chosen randomly from all treated groups (12 eggs / group / incubation times) were weighed and opened then the embryos were separated from the remaining egg contents. Embryos were rinsed in saline and blotted dry on an absorbent paper to remove excess moisture. Dried embryos were allowed to reach room temperature and then weighed to the nearest 0.001 g. Relative embryo weight, yolk sac weight and eggshell weight were determined as a percentage of egg weight, and eggshell thickness was measured using the Micrometer. Eggs that failed to hatch and having full opportunity to hatch were broken out and examined macroscopically to estimate the embryonic development and assigned according to their times of death by days if possible, embryonic mortality classified into three periods (1-7 ,8-12 , 13-18, 1-18 and 18-20 days). Also, pipped eggs and embryonic malposition

were estimated. Hatchability (%) was calculated as a percentage of the number of hatched sound chicks to number of fertile eggs. At hatch, day-old chicks from each group were weighed. Also, chick length was measured from the tip of the beak to the end of the middle toe with the chick' s dorsal surface extended over a ruler. Moreover, chick quality measurements (activity, downs and appearance, retracted yolk, eyes, navel area and remaining membrane) were assessment using the Tona scoring system (Tona *et al*., 2003).

Hematological parameters, Biochemical constituents, Yolk sac, and Internal organs' weight of hatched chicks

At hatch, according to each group, 6 hatched chicks (24 chicks) were humanely slaughtered to obtain blood samples in EDTA tubes. Part of the fresh blood samples were taken to measure the hemoglobin (Hb) content, red blood cells (RBC's) counts, white blood cells (WBC's) counts, lymphocyte (L) % , heterophil (H) % ,platelets and packed cell volume (PCV). The remaining portion of the blood samples were centrifuged at 3000 rpm for 20 minutes to obtain plasma samples, which were stored at ~ 20°C until analysis. Plasma total proteins (g/dl), albumen (g/dl), globulin (g/dl), glucose (mg/dl), high density lipoprotein (HDL) (mg / dl), low density lipoprotein (LDL) (mg/dl), total antioxidant capacity (TAC) (mmol/ml) , malondialdehyde (MDA) (nmol/ml) , triiodothronine (T_3) (ng/ml) and thyroxin (T_4) (ng/ml) were determined spectrophotometrically using available commercial Kits (Diamond Diagnostics Chemical Company, Egypt).

After blood collection, yolk sac, gizzard, heart, liver, intestine, stomach, fabricius gland, spleen and gall bladder were weighted. Also, the right and left tibia were removed and weighed to the nearest 0.1g. Relative weights (percentage per chick weight) were determined.

Gene expression by real-time PCR

Tissue sample collection.

At one day of age, six chicks were slaughtered from each group randomly selected to be used for the sample collection. Heart tissue specimens were collected and snapped frozen in liquid nitrogen for gene expression assessment from each bird.

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR) Analysis

Heart tissue samples were ground by using a tissue homogenizer (Tissue Grinder Mixy, NIPPON Genetics EUROPE GmbH), followed by total RNA extraction using Trizol reagents and treated with RNAse-Free DNase Set (Qiagen, Valencia, CA) according to the manufacturer's instructions. The RNA was suspended in DEPC water, and sample purity and concentration were measured on a spectrophotometer (pg T80, UK), and stored at - 86 ◦C. The concentration of the extracted RNA was assessed by UV absorbance, and the $OD_{260/280}$ ratios for all samples were >1.9. Complementary DNA (cDNA) was synthesized from RNA using ABT H-minus MMLV cDNA synthesis kit (Catalogue number; ABT009, Applied Biotechnology, Egypt). Complementary DNA (cDNA) samples were diluted 1:1 prior to RT-PCR analysis. Each reaction consisted of 1 µL diluted cDNA, 0.4 µL forward primer (10 µM), 0.4 µL reverse primer (10 µM), 3.2 µL DEPC water, and 5 µLTransStart® Green qPCRSuperMix (TransGen biotech co., LTD, China). Primer sequences used for the RT-PCR assays are listed in Table 1. The qPCR conditions were 94 ◦C for 30 s, followed by 45 cycles of 94 ◦C for 5 s, 60 ◦C for 15 s, and 70 ◦C for 10 s. In addition, at the end for each reaction, a melting temperature curve of each qPCR reaction was determined. Each biological sample was run in triplicate using Rotor gene Q (Qiagen, USA). The quantification of the target gene was normalized to the housekeeping gene

beta actin (β actin). The mRNA relative expression level was expressed as the copy concentration of the target gene divided by that of the endogenous reference (target gene/β actin) **(**Lu *et al*., 2008).

The gene expression in the form of relative quantification was estimated using Ct values. The fold change of target genes in comparison to control was calculated according to livak method $2^{-\Delta\Delta}$Ct (Livak and Schmittgen, 2001).

Statistical analysis

This study used an entirely statistical randomization design according to Snedecor and Cochran (1982). All results were subjected to standard statistical one-way analysis of variance (ANOVA) in the Statistical Package for the Social Sciences, version 20 (SPSS, 2011).

The statistical model used was as follows:

$$X_{ij}= \mu +T_i+ e_{ij}$$

Where x_{ij} is the value of the measured variable, μ is the overall mean, T_i is the effect of treatment (i= 4 treatments), and e_{ij} is the random error.

Duncan's multiple range test was implemented to evaluate whether the means of the variables differed significantly or not (Duncan, 1955). Means were considered statistically significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Effect of *in ovo* injection with *Spirulina Platensis*

1. Egg weight loss percentages:

As clarified in Table 2, there were no significant differences between all experimental groups with respect to egg weight loss % during 0-12 days of incubation. Injecting eggs with SP at different concentrations (0.2 and 0.4 mg/egg) on 14th d of incubation during thermal manipulation led to a significant decrease($P \leq 0.05$) in egg weight loss (%) when compared to sham control and with a non-significant reduction compared to control group among the experimental incubation periods (12-18 d and 0-18 d). Moreover, injecting the eggs with the highest concentrations of SP represented the lowest egg weight loss (%), followed by group of SP at 0.2 mg/egg as compared with control groups.

Apparently from data of this table that the effect of *in ovo* injection during thermal manipulation did not appear during interval (0-12d) as thermal manipulation was done starting from day 12th of incubation and *in ovo* injection was at 14th d of incubation, and the reduction in egg weight loss (%) for groups of supplementation the embryos with SP solutions could be due to carotenoids present with high concentration in SP which in turn work to decrease the oxidation of yolk lipids (Assuncao *et al*., 2021), where the oxidation of yolk lipids produces metabolic water that is added to the total volume of the egg (Molenaar *et al*., 2010). Moreover, Yalcin and Siegel, (2003) determined that 10-12% weight loss is necessary during incubation in order to get a good incubation results. Therefore, if water is continuously diffusing out of the egg during incubation, some water must also be produced by the embryo to prevent excessive water loss. The same opinion was held by Ebtsam *et al*. (2018) claimed that by supplementing the diet with SP significantly reduced egg weight loss % compared to control group.

2. Embryonic development percentages:

Data in Table 3 showed that embryonic development at 18th day of incubation was significantly ($P \leq 0.01$) advanced during thermal manipulation by *in ovo* feeding of SP at 0.4 mg /egg compared to control groups and *in ovo* injection with 0.2 mg /egg. Relative yolk sac was significantly decreased in the incubated eggs injected with 0.4 mg SP/egg compared to the control groups. Relative

shell weight and shell thickness were not affected by *in ovo* injection with SP.

The present results are in harmony with previous research works which demonstrated the role of SP for improving metabolic processes and cellular differentiation of embryos during embryonic developments (Sporn *et al*., 1994). Also, SP is a good source of antioxidants such as carotenoids (β-carotene, zeaxanthin chlorophyll, tocopherol, lutein and lycopene (Assuncao *et al*., 2021), many vitamins as C, E, A, D(Ghaeni and Roomiani 2016). Carotenoids work to protect fetal tissues from oxidative stress because they have antioxidant properties (Blount *et al*., 2000). In addition, Wei *et al*. (2024) demonstrated that vitamin D_3 stimulating the differentiation of cells, increase the activity of the cell in the intestinal mucosa and stimulates RNA for protein synthesis. Also, Vitamin E enhance antioxidant status of the eggs (Surai, 2000) and protect eggs from against oxidation (Puthpongsiriporn *et al*., 2001) and this cause increase body weight. Moreover, Schaal, (2008) attributed that the injected eggs with SP produce a large amount of energy, which in turn works to prevent the harmful effects of hydro peroxides, thus improve the embryonic development. Supporting to the previous results, many researchers as (Mirzaie *et al*. 2018; Khan *et al*. 2020 ; Moustafa *et al*. 2021; Abdel-Moneim *et al*. 2021) have praised the role of SP as an antioxidant that improves antioxidative biomarkers of heat-stressed birds. Furthermore, Ebtsam *et al*.(2018) observed that embryonic weight at 18^{th} of incubation was significantly increased by supplementing the diet with SP. El-Shall *et al*. (2023) proved that SP improve growth rates especially under stress conditions.

3. Hatching event:

The results achieved in Table 4 indicated that injecting SP solutions into yolk sac at different concentrations led to a significant reduction in embryonic mortality (%) during (13-18d), (1-18d) and (18-20d) of incubation and the same reduction ($P \leq 0.01$) was found for embryonic malposition (%) compared to control groups. Moreover, the group of injected 0.4 mg SP/egg recorded lowest embryonic mortality % and pipped eggs % compared to all experimental groups except that for group of injection 0.2 mg SP/egg with respect to pipped eggs %. It is evident from current outcomes that groups of SP *in ovo* injection with different concentrations recorded the highest significant ($P \leq 0.01$) percentage of hatchability of fertile eggs compared with control groups. Also, in *ovo* feeding with the highest concentration of SP was the best for increasing hatchability (%).

It is evident from the results of the previous Table that the reduction of embryonic mortality percentage throughout (13-18d) and (18-20 d) of incubation , embryonic malposition and pipped eggs % in groups of eggs that injected with SP especially for the highest concentrations compared to control groups during thermal manipulation could be due to the effective role of SP as a powerful antioxidant (Pestana *et al*. 2020). Based on the results presented herein, it appears that the components found in SP that give its antioxidant properties, such as carotenoids and vitamins (Vit.E ,Vit.B_2) protect the embryos during thermal manipulation by decreasing concentrations of oxidative stress, free radicals and metabolites of reactive oxygen, hence reducing the damage in cellular membranes and embryonic mortality (Surai, 2012 and Duarte *et al*., 2015). Also, Foye *et al*. (2006) found that injecting eggs with natural antioxidants is one of the most suitable ways to reduce stress and fetal mortality and improve immunity. At the same trend, Ismail *et al*. (2019) found that eggs of Sinai hens injected with 2.5 mg SP /egg reduced embryonic mortality

compared to control groups. In addition, *in ovo* feeding with Vit E and Vit B_2 as antioxidants decreased late embryonic mortality (Ebrahimi *et al*., 2012 and Kalaba *et al*., 2024). Different researches confirmed our results regarding the role of SP for improving rate of hatch, Adriana *et al*. (2005) mentioned that SP rich in vitamin D, which changes cloning rate of target genes that responsible for the biological response, hens regulates cell growth and increased hatchability (%). Also, Kotrbaček *et al*. (2013), Selim *et al*. (2018) , Aljumaily and Taha, (2019) indicated that *in ovo* injection with different concentrations of SP solution in late stages of incubation improved immunity of embryos by decreasing oxidative stress and redirect the available energy towards the hatching process, thus improving hatchability. Furthermore, there are many researchers who attributed the role of SP in improving hatchability (%) to its antioxidant activity due to it containing phenolic compounds and natural pigments like β-carotene, β-cryptoxanthin, chlorophyll, echinenone, myxoxanthophyll, phycoerythrin, phycocyanin, xanthophylls, and zeaxanthin, which in turn work to protect the embryos from free radicals and improve the oxidative state of the embryos during thermal manipulation (Gad *et al*. 2011; Zaid *et al*. 2015; Asghari *et al*. 2016; Hajati and Zaghari, 2019 and Hajati *et al*.,2021). On the other hand, Taher *et al*. (2024) showed that injecting hatching eggs with 0.5 ml/egg (contains 3 mg of SP extract) decreased hatching rate. With respect to hatchability (%), the aim of this study is to face the bad influence of increasing incubation temperature (heat stress) during hatchability process had been done by *in ovo* feeding embryos with SP solutions, especially for the highest concentrations compared to control groups.

4. Quality of hatched chicks:

In ovo supplementation with SP solution at a dose of 0.2 and 0.4 mg /egg during thermal manipulation had significant effect on weight and quality of the newly hatched chicks ($P \leq 0.01$) compared to control groups, although a tendency to increase these parameters in SP 0.4 mg/egg group can be noted (Table 5).

The positive influence of *in ovo* SP injection on chick weight and quality could be attributed to the increase of embryonic weight as found in Table 3 as a result of increasing nutritional value of SP, As it contains several biologically active compounds such as antioxidants, vitamins and minerals (Farag *et al*.,2016), which protect the embryo from harmful effects resulting from increasing the temperature during incubation and obtaining a chick of good weight, ideal condition and high quality. Supporting to results and interpretation herein, Swanson *et al*. (2012) observed that the increase in chick weight is due to the fact that fatty acids, mainly linoleic acids, found in SP, have the ability to transform into prostaglandin, eicosapentaenoic acid, and docosahexaenoic acid, which in turn stimulate the growth of the embryos and prevent them becoming sick. Besides, Ismail *et al*. (2019) found significant differences in hatched chicks weight by injecting eggs of Sinai hens with different concentrations of *Spirulina algae*. In quail eggs, injections with different concentrations of SP led to an increase in the weight of hatched chicks and improved their health status by improving the oxidative state of the embryos (Hajati *et al.*, 2021). Moreover, Kalaba *et al.* (2024) indicated that *in ovo* feeding with vitamins E, B_1, and B_2 increased chicks weight at hatch in comparison to positive control. These results are alarming to researchers Aljumaily and Taha, (2019), who found that injecting quail eggs with different concentrations of *Spirulina* has no

significant effect on hatched chicks weight. Furthermore, the positive effect of *in ovo* SP injection on chick quality compared with control could be related to the fact that SP riched in Vit.E which increase the oxidative status of chicks (Araujo *et al*. 2019).

5. Hematological parameters of the day-old chicks:

Regards to Table 6, *In ovo* administration of different concentrations of SP solutions on 14th d of incubation during thermal manipulation showed significantly ($P \leq 0.01$) increased the values of blood Hb, platelets and WBC's counts, while, reduced ($P \leq 0.01$) the values of H/L ratio for baby chicks compared to control groups. Moreover, lymphocytes (%) and RBC's counts values were increased ($P \leq 0.01$) as a result of SP injections, when compared to sham control group. In contrast, *in ovo* administration during heat treatment had no significant effect for values of heterophils (%) and PCV (%). This results might be due to the affluent *Spirulina* with vitamins, macrominerals, microminerals (especially Fe, Cu, Zn), ß-carotenes, chlorophyll, tocopherols, phycocyanins, amino acids and gamma linoleic acid (Khan *et al*.,2005), which plays essential role as antioxidants materials and prevent oxidative stress, Hence promoted humeral immune system. Contradicted reports regarding this research point are documented by Attia *et al*. (2023) who noted that iron in *Spirulina* plays a vital role in biosynthesis of hemoglobin and RBC's, which is necessary for metabolic enzymes synthesis as cytochromes superoxide. Also, Ismail *et al*. (2019) found significant increase in blood Hb, lymphocytes and WBC's for chicks produced from injected eggs with 5.0 mg SP/eggs as compared with positive and negative control groups. In addition, Ebtsam *et al*.(2018) proved that dietary supplementation with varied concentrations of *Spirulina* increased values of RBC's , Hb,WBC's and lymphocytes , Whereas, H/L % was decreased among all treated groups compared to control. Furthermore, Hajati and Shokouri, (2023) concluded that feeding microencapsulated *Spirulina* powder promoted humeral immune system and increased the percentage of red blood cells in broiler chickens . Also, the non-significant increase in PCV by *Spirulina* supplementation was indicated by Shanmugapriya *et al*. (2015).

6. Blood parameters of hatched chicks:

The average concentrations of selected biochemical indices of plasma hatched Silver Sabahia chicks are shown in Table 7. Experimental administration of the highest concentration of SP (0.4 mg SP/egg) *in ovo* injection increased ($P \leq 0.01$) the value of total proteins and globulin of day-old chicks compared to control groups and SP dose of 0.2 mg/egg, besides that, there were no significant differences between control and sham control groups. Moreover, chicks resulting from eggs that were injected with different concentrations of SP had highest ($P \leq 0.01$) values of plasma glucose, HDL, TAC, T_3 and T_4 compared with those for control groups ones. Conversely, levels of plasma malondialdehyde was significantly decreased in the same mentioned groups compared to control groups. Also, there were no significant differences between all experimental groups with respect to plasma albumin and T_3/T_4 ratio.

It is worth noting that a general improvement in body function of hatched chicks was observed in group injected with 0.4 SP/egg and followed by group of 0.2 mg SP/egg, which was expressed in higher values of total proteins, glucose, HDL, TAC, T_3 and T_4 with lower values of LDL and MDA of day old chicks. In the aforementioned groups, high concentrations of plasma

proteins and globulin which are important in maintaining the homeostasis of body (Azhar *et al*.,2024), and this increase may be due to SP containing Vitamin E,B_1 and B_2 (Ross and Dominy, 1990), which in turn works to strengthen the resistance of the embryo's tissues to the oxidation of proteins that can be caused by exposure to high temperature (Surai *et al*.,2016). This is confirmed by the study of Kalaba *et al*.(2024), who reported an increase in plasma total protein of chicks by injecting egg with vitamin E,B_1 and B_2. Also, Ismail *et al*. (2019) stated that injecting embryos chicken egg with *Spirulina algae* led to increase significant in the levels of globulin and total protein. Regarding of a tendency of increase in plasma glucose level by SP *in ovo* injection may be attributed to that SP contains many vitamins, especially B_1 and B_2 which acts as a cofactor for multiple enzymes that help break down dietary carbohydrates and convert them into glucose. Similar results were reported by Kitakoshi *et al*. (2007) who showed that *in ovo* injection with B_2 increased values of glucose for hatched chicks. Also, Bjonnes *et al*. (1987) and John*et al*. (1987) reported that Vit. B_1 may increase glucose level by stimulating the gluconeogenesis or glycolysis of stored glycogen. Furthermore, Christensen *et al*., (2001) suggested that level of blood glucose is important at a late embryonic stage of hatching and immediately post-hatching. Towards the termination of the hatching period, embryos draw on reservoir energy to cover the increased glucose requirements during incubation period. Also, the present results of the significant increase of HDL and decrease of LDL due to SP are coincided with the results of Yeum and Russell, (2002) who mentioned that beta-carotene in *Spirulina* has a hypocholesterolemic effect and seems to displace cholesterol in the transport of lipoproteins and released into circulation (Salma *et al*., 2007). In addition, there was many researchers who have confirmed the role of SP in increasing HDL and lowering LDL when added to feed (Mariey *et al*. (2014) ; Mobarez *et al*. (2018) ; Abd El-Hady and El-Ghalid (2018)).

As for the increase in the plasma level of antioxidants with the decrease in MDA for the *Spirulina* injection groups, it was due to the antioxidative action of SP (Aljumaily and Taha , 2019), In spite of the action of temperature increase for embryos. These results coinicided with the results reported by Rocha *et al*. (2013) and Karadas *et al*. (2016) who found that carotenoids are among the natural lipids and antioxidants found in *Spirulina*, which may improve total antioxidant capacity available to the embryo and baby chicks. Also, Ebtsam *et al*. (2018) concluded that SP supplementation significantly increased values of plasma total antioxidant and reduced values of plasma MDA for blood of baby chicks compared to control group. Also, Hajati *et al*. (2021) reported that feeding embryos with SP can improve antioxidant in chicks of quail and thermotolerance of embryo during last days of incubation. In addition to, Taher *et al*. (2024) observed that inject the egg with organic selenium and *Spirulina* reduced the MDA in the blood serum. Also, the increase of the studied hormones in the chicks resulting from egg injection groups with SP could be mainly due to the double cicks of thermal manipulation and *in ovo* injection. Thyroid hormones are involved in the regulation of metabolic pathways of all nutrients (EL-Sahn *et al*., 2024). Another explanation, Kalaba *et al*. (2024) demonstrated that injection the eggs with Vit. E, B_1 and B_2 increased ($P < 0.05$) plasma T_3 and T_4 concentration of chicks compared with control group .

7. Relative weight of yolk sac and internal organs of the day-old chicks:

Data of the impact of *in ovo* supplementation with SP during thermal manipulation on the relative weights of internal organs for hatched chicks are displayed in Table 8. The results showed a significant ($P \leq 0.01$) decrease in weight of yolk sac (%) for SP injection groups with different concentrations (0.2 and 0.4 mg SP/egg) compared to control groups. In contrast, the relative weight of liver was increased significantly ($P \leq 0.01$) as a result of the injection of eggs with SP compared to the previously mentioned groups. While, the treatments in this experiment had no significant influence on the rest of the internal organs weights for hatched chicks (%).

In the current study, the significant decrease in the relative weight of yolk sac as a result of SP injection coincides with an increase in fetal growth in Table 3, and this due to the increased fetal consumption of yolk sac. As for the increase in liver weight (%) for the same groups, it might due to the liver's role in regulating of yolk sac lipid metabolism during the critical embryo to hatchling transition (Cogburn *et al*., 2018). While, the absence of any significant differences between the groups for the rest of the relative weights of internal organs can be considered as evidence that concentrations used of SP do not have any negative effects on the embryos. These results coincided with the results reported by Ismail *et al*. (2019) who concluded that *in-ovo* injection with 5.0 mg SP/egg increased the relative weight of chicks liver. Furthermore, Hajati *et al*. (2021) stated that the yolk sac (%) in quail chicks resulting from eggs previously injected with SP at 2.5 or 3.5mg /egg was lower than control groups and this decrease was explained by the fact that *Spirulina* is a microalgae that works to increase the beneficial microorganisms present in the intestines and improve digestion and absorption processes (Seyidoglu *et al*. 2017). Whereas, Abouelezz, (2017) and Aljumaily and Taha, (2019) demonstrated that injection of the embryos with *Spirulina platensis* did not affect the relative weights of internal organs for hatched chicks.

8. Gene expression ratio of HSP70 in heart tissues of the day-old chicks:

The expression of gene HSP70 in Sliver Sabahia chicks strain heart tissues as shown in Fig.1. At one day of age, HSP70 was increased expressed in chicks of injection group with 0.2 mg SP/egg, while group of 0.4 mg SP/egg was most highly expressed ($P < 0.001$) compared with the control and sham control groups. No significant differences between the control and sham control groups were observed. The present results are in agreement with (Yu *et al*., 2021) who found that heat stress significantly increased the expression of HSP70 and reduced the expression of HSP70 mRNA in the duodenum. The increase of expression level of HSPs protect against the heat shock adverse subsequent tissue injuries (Akbarian *et al.*, 2016). This result is a strong indication of increased protective regulatory mechanisms of gene heat shock protein (HSP70) that play a key role in protecting and repairing cells and tissues during heat stress when using *Spirulina* as a functional organic positive effect on heat stress (Alaqil and Abbas, 2023). In addition, Elbaz *et al*. (2022) and Abdel-Moneim *et al*. (2022) indicated that *Spirulina* plays an important role in strengthening the immune system to fight heat stress through its action as an antioxidant.

CONCLUSION

Thermal manipulation during incubation has positive effect on the thermotolerance of birds during summer season. *In ovo* injection with *Spirulina* solution (0.4 mg/ml) accompanied with thermal manipulation during

embryogenesis could be considered as double kicks to benefit from the advantages of thermal manipulation and avoid its negative effects and thus improving embryonic development, hatchability percentage, chick quality, physiological performance besides increasing the expression of gene HSP70 in heart tissue of Sliver Sabahia chicks, where, HSP70 gene has an important role in enhancing thermotolerance, which is essential for developing genetic improvement programs with leveraging heat resilience in the context of global climate change.

Table (1): Primer Information

Gene	Sequence accession ID	Product size (bp)	Forward/Reverse primer (5′-3′)
HSP70	NM_001006685.1	20	CTCTGAGCTCTTCCACGCAA
			GCTAAGGCGACCCTTGTCAT
B-actin	NM_205518.1	125	TCCCTGGAGAAGAGCTATGAA
			CAGGACTCCATACCCAAGAAAG

Table (2): Effectof *in ovo* injection with *Spirulina platensis* solutions during thermal manipulation on egg weight loss % of Sabahia Silver chicken strain

Traits	Control	Sham Control	SP (mg/egg)		SEM	P value
			0.2	0.4		
Initial egg weight (g)	51.62	51.75	51.59	51.65	0.57	0.998
Egg weight at 12 d (g)	47.62	47.69	47.65	47.82	0.57	0.995
Egg weight at 18 d (g)	45.81	45.53	46.11	46.41	0.57	0.730
Egg weight loss (0 -12 days) %	7.76	7.82	7.66	7.43	0.33	0.853
Egg weight loss (12 -18 days) %	3.79^{ab}	4.58^{a}	3.23^{b}	2.94^{b}	0.28	0.001
Egg weight loss (0 -18 days) %	11.26^{ab}	12.04^{a}	10.64^{b}	10.16^{b}	0.41	0.015

[ab] Means in the same row having different superscripts are significantly different ($P \leq 0.05$). SEM: Standard error of the means. P value: Probability level, d: day.

Table (3): Effectof *in ovo* injection with *Spirulina platensis* solutions during thermal manipulation on embryonic development of Sabahia Silver chicken strain

Traits	Control	Sham Control	SP (mg/egg)		SEM	P value
			0.2	0.4		
At 12thd of incubation						
Egg weight (g)	47.59	47.67	47.64	47.62	0.99	1.000
Embryonic weight (%)	20.36	20.35	20.42	20.59	0.28	0.945
Yolk sac (%)	60.69	60.53	60.48	60.30	0.26	0.848
Shell weight (%)	10.56	10.71	10.65	10.73	0.19	0.940
Shell thickness (mm)	0.36	0.36	0.37	0.36	0.00	0.971
At 18thd of incubation						
Egg weight (g)	45.69	45.54	45.46	45.57	1.78	1.000
Embryonic weight (%)	49.69^{bc}	47.41^{c}	51.77^{b}	58.05^{a}	1.10	0.001
Yolk sac (%)	27.96^{ab}	29.93^{a}	25.54^{bc}	23.57^{c}	0.843	0.013
Shell weight (%)	9.32	9.28	9.33	9.22	0.12	0.913
Shell thickness (mm)	0.34	0.34	0.35	0.34	0.01	0.941

[abc] Means in the same row having different superscripts are significantly different ($P \leq 0.05$). SEM: Standard error of the means. P value: Probability level.

Table (4): Effectof *in ovo* injection with *Spirulina platensis* solutions during thermal manipulation on hatching event of Sabahia Silver strain eggs

Traits	Control	Sham Control	SP (mg/egg)		SEM	P value
			0.2	0.4		
Embryonic mortality %						
1- 7 d	1.83	1.90	1.80	1.86	0.08	0.881
8-12 d	1.94	1.96	1.94	1.94	0.26	1.000
13-18 d	5.98^{a}	5.94^{a}	3.33^{b}	0.64^{c}	0.33	0.000
1-18 d	9.77^{a}	9.80^{a}	7.08^{b}	0.44^{c}	0.50	0.000
18-20 d	6.16^{a}	6.39^{a}	3.78^{b}	1.22^{c}	0.30	0.000
Pipped eggs (%)	3.28^{a}	3.66^{a}	2.67^{ab}	1.33^{b}	0.40	0.034
Hatchability of fertile egg (%)	80.80^{c}	80.17^{c}	86.50^{b}	93.00^{a}	0.78	0.000
Embryonic malposition (%)	4.57^{a}	4.61^{a}	2.09^{b}	1.52^{b}	0.37	0.001

^{abc} Means in the same row having different superscripts are significantly different ($P \leq 0.05$). SEM: Standard error of the means. P value: Probability level, d: days.

Table (5): Effect of *in ovo* injection with *Spirulina platensis* solutions during thermal manipulation on the quality of hatched chicks

Traits	Control	Sham Control	SP (mg/egg)		SEM	P value
			0.2	0.4		
Chick weight (g)	34.74^{c}	32.58^{d}	35.86^{b}	36.88^{a}	0.26	0.000
Chick length (cm)	14.11^{c}	13.76^{d}	14.97^{b}	16.00^{a}	0.10	0.000
Activity (good)	91.60^{c}	90.25^{c}	95.87^{b}	99.60^{a}	0.63	0.000
Downs and appearance (Clean and dry)	93.70^{c}	91.93^{c}	97.03^{b}	99.80^{a}	0.66	0.000
Retracted yolk (Body with normal swallowed yolk)	97.70^{b}	95.62^{c}	100.00^{a}	100.00^{a}	0.28	0.000
Eyes (Opened and bright)	92.93^{b}	91.18^{b}	97.90^{a}	99.00^{a}	0.65	0.000
Navel (Completely closed and Clean)	92.50^{b}	92.28^{b}	97.90^{a}	99.07^{a}	0.40	0.000
Remaining membrane (No membrane)	94.17^{c}	91.40^{d}	97.60^{b}	99.47^{a}	0.44	0.000

^{abcd} Means in the same row having different superscripts are significantly different ($P \leq 0.05$). SEM: Standard error of the means. P value: Probability level.

Table (6): Effects of *in ovo* injection with *Spirulina platensis* solutions during thermal manipulation on hematological parameters of the day-old chicks

Traits	Control	Sham Control	SP (mg/egg)		SEM	P value
			0.2	0.4		
Hb (g/dl)	10.48^{b}	10.06^{b}	11.46^{a}	11.64^{a}	0.18	0.000
RBC's(10^6/mm^3)	2.32^{bc}	2.03^{c}	2.60^{b}	3.15^{a}	0.11	0.000
WBC's(10^3/mm^3)	8.96^{c}	8.80^{c}	9.57^{b}	11.09^{a}	0.17	0.000
Lymphocytes (%)	68.20^{b}	63.40^{c}	69.60^{ab}	73.40^{a}	1.21	0.001
Heterophils (%)	24.20	27.22	23.60	23.20	1.01	0.059
H/L ratio	0.42^{a}	0.43^{a}	0.34^{b}	0.32^{b}	0.02	0.002
Platelets(10/uL)	13.20^{b}	11.80^{b}	17.40^{a}	18.60^{a}	1.24	0.004
PCV (%)	34.00	33.56	35.60	37.00	1.10	0.188

^{abc} Means in the same row having different superscripts are significantly different ($P \leq 0.05$). SEM: Standard error of the means. P value: Probability level, Hb: Hemoglobin concentration, RBC's: Red blood cells, WBC's: White blood cells, H/L: Heterophils/ Lymphocytes, PCV: Packed cell volume.

Table (7): Effects of *in ovo* injection with *Spirulina platensis* solutions during thermal manipulation on biochemical parameters of hatched chicks

Traits	Control	Sham Control	SP (mg/egg)		SEM	P value
			0.2	0.4		
Total Proteins (g/dl)	5.28^{b}	5.09^{b}	5.29^{b}	5.67^{a}	0.09	0.003
Albumin (g/dl)	3.55	3.61	3.34	3.33	0.11	0.211
Globulin(g/dl)	1.72^{bc}	1.48^{c}	1.95^{b}	2.34^{a}	0.08	0.000
Glucose (mg/dl)	159.00^{b}	147.40^{c}	171.00^{a}	167.60^{a}	1.55	0.000
HDL (mg/dl)	31.60^{c}	31.24^{c}	37.60^{b}	41.04^{a}	0.53	0.000
LDL (mg/dl)	17.60^{b}	19.00^{a}	16.20^{c}	15.81^{c}	0.26	0.000
TAC (mmol/ml)	2.53^{c}	2.05^{d}	3.65^{b}	4.72^{a}	0.10	0.000
MDA(nmol/ml)	4.14^{b}	4.91^{a}	3.23^{c}	2.48^{d}	0.17	0.000
Triiodothyronine (T3) (ng/ml)	1.08^{b}	1.08^{b}	1.35^{a}	1.37^{a}	0.07	0.014
Thyroxin (T4) (ng/ml)	6.61^{b}	6.56^{b}	7.40^{a}	7.74^{a}	0.23	0.007
T_3/T_4 ratio	0.16	0.16	0.18	0.18	0.01	0.503

^{abc} Means in the same row having different superscripts are significantly different ($P \leq 0.05$). SEM: Standard error of the means. P value: Probability level. HDL: High density lipoprotein, LDL: Low density lipoprotein, TAC: Total antioxidant capacity, MDA: Malondialdehyde .

Table (8): Effect of *in ovo* injection with *Spirulina platensis* solutions during thermal manipulation on relative weight of yolk sac , some internal organs, and tibia of the day-old chicks

Traits	Control	Sham Control	SP (mg/egg)		SEM	P value
			0.2	0.4		
Yolk sac (%)	15.82[a]	17.41[a]	11.19[b]	10.83[b]	0.52	0.000
Gizzard (%)	4.78	4.58	4.96	4.99	0.20	0.512
Heart (%)	0.65	0.63	0.65	0.67	0.02	0.165
Liver (%)	2.13[b]	2.03[b]	2.75[a]	2.78[a]	0.15	0.003
Intestine (%)	3.99	3.94	4.00	4.13	0.20	0.925
Stomach (%)	0.67	0.72	0.81	0.85	0.06	0.153
Fabricius gland (%)	0.18	0.15	0.15	0.14	0.03	0.838
Spleen (%)	0.08	0.06	0.04	0.05	0.01	0.222
Gall bladder (%)	0.16	0.15	0.14	0.13	0.02	0.765
Right tibia (%)	0.62	0.51	0.64	0.66	0.05	0.175
Left tibia (%)	0.57	0.56	0.59	0.63	0.05	0.728

[ab] Means in the same row having different superscripts are significantly different ($p \leq 0.05$). SEM: Standard error of the means. P value: Probability level.

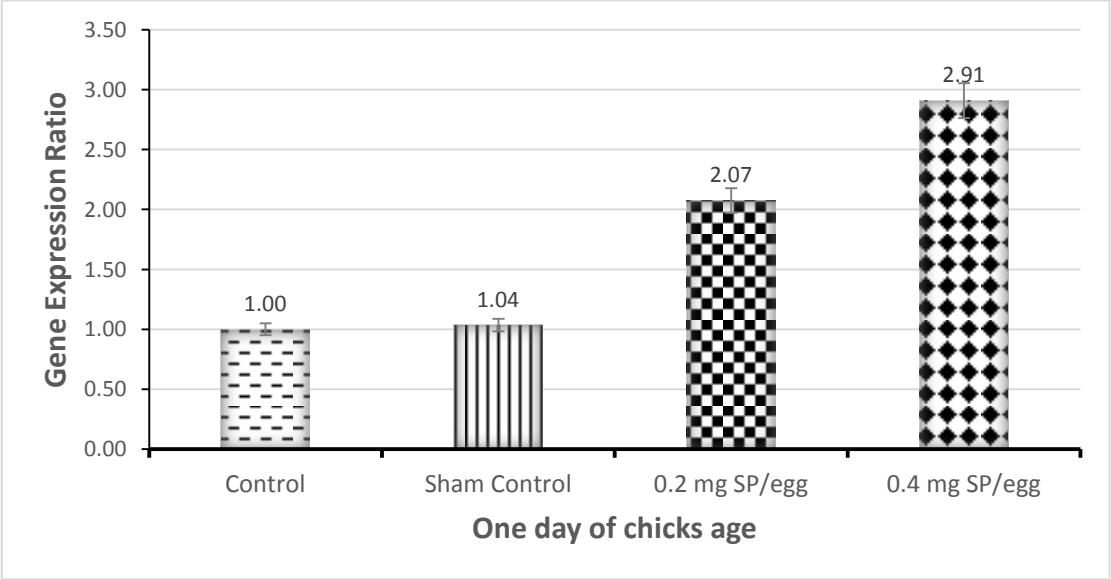

Figure (1): Gene expression ratio of HSP70 in Sliver Sabahia chicks strain heart tissues at one day of age. Control, Sham control, 0.2 mg SP/egg and 0.4 mg SP/egg. Means with different superscripts represent significant differences between treatments at same time ($p \leq 0.01$).

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الملخص العربى

تأثير حقن البيض بالأسبيرولينا فى تخفيف الأثار السلبية للمعالجة الحرارية أثناء النمو الجنينى لسلالة دجاج الصبحية فضى

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تمت هذه الدراسة لتسليط الضوء على تأثير حقن البيض بمحاليل سبيرولينا بلاتنسيس (SP) للتخفيف من الأثار السلبية للمعالجة الحرارية أثناء التحضين .و تمت دراسة تأثير الحقن بالأسبيرولينا على التطور الجنينى ، وصفات الفقس ، والأداء الفسيولوجى والتعبير عن الجين HSP70. تم استخدام اجمالى750 بيضة تفريخ من سلالة دجاج الصبحية الفضى عمر 45 اسبوعا . تم تحضين البيض عند 37,5 °م (F°99,5) و رطوبة نسبية 55% من 1 الى 11 يوم من التحضين . ثم تم تعريض الأجنة الى المعالجة الحرارية يوميا عند 39,5°م (F°103,1) ورطوبة نسبية 60% لمدة أربع ساعات من اليوم ال12-18 من التحضين . فى اليوم الرابع عشر من التجضين ، تم التوزيع العشوائى لعدد 660 بيضة مخصبة بها أجنة حية عشوائيا على أربع مجموعات ، 165 بيضة لكل مجموعة (3 مكررات ، 55 بيضة لكل مكررة) ، المجموعة الأولى :بيض بدون حقن وتم تقديمه كمجموعة كنترول ، المجموعة الثانية : بيض تم حقنه بمحلول ملحى وتم تقديمه كمجموعة كنترول زائف (1مل محلول ملحى / بيضة)، المجموعة الثالثة : بيض تم حقنه ب 0,2 مجم SP/بيضة ،المجموعة الرابعة : بيض تم حقنه ب 0,4 مجم SP/بيضة. أظهرت النتائج أن حقن البيض ب SP بتركيزات مختلفة (0,2 أو 0,4 مجم /بيضة) أدى الى انخفاض الفقد فى وزن البيض (%) و كيس الصفار (%) والنفوق الجنينى ($P \leq 0.01$) والبيض الناقر (%) و الأوضاع الجنينة الشاذة ($P \leq 0.01$) مقارنة بمجموعات الكنترول .بينما تحسن معنويا ($P \leq 0.01$) كل من النمو الجنينى و نسبة الفقس ووزن و جودة الكتاكيت و معظم قياسات الدم و التعبير عن الجين HSP70 مقارنة بمجموعات الكنترول . كانت أفضل النتائج من خلال تغذية البيض على SP بتركيز 0,4 مجم SP/بيضة .

الخلاصة ، تغذية البيض بمحلول سبيرولينا بتركيز 0,4 مجم SP/بيضة أثناء المعالجة الحرارية أدى الى تخليق أنظمة هرمونية و مناعية جديدة و زيادة مستوى التعبير الجينى HSP70 فى الأجنة مع قدرة أكبر على تحمل ارتفاع درجة الحرارة داخل المفرخة دون أى تأثير ضار ، وقد انعكس هذا على تحسين صفات الفقس و جودة الكتاكيت .