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Effect of *in ovo* injection of epigallocatechin-3 gallate and oleuropein on hatching, productive and physiological aspects of broiler chicks exposed to short heat stress

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ABSTRACT

This study was aimed to investigate the influence of *in ovo* injection (IO) into air cell (AC) or yolk sac (YS) of epigallocatechin-3 gallate (EG) and oleuropein (OL) as antioxidants on hatching, physiological and productive performance of heat-stressed chicks. 840 fertile eggs were chosen for IO on 12th day of incubation. Eggs were divided into 7 groups within 4 replications each as follows: negative control (NC) without IO and other groups involved IO of 100 µl distilled water and 5 mg each of EG and OL in both AC and YS sites of egg. After hatching, chicks were exposed to heat stress for 24 h and raised for 42 d. Compared with NC, the results revealed that main effect of IO with EG and OL increased ($p \leq 0.05$) hatchability and decreased deformed chicks and serum levels of glucose, cholesterol, aspartate aminotransferase, corticosterone and heat shock protein 70 with enhancing feed efficiency, superoxide dismutase, glutathione peroxidase and thyroxine in serum or liver. High chick length and relative chick weight with low serum protein carbonyl and feed intake were recorded ($p \leq 0.05$) by EG. Low rectal temperature and heterophil to lymphocyte ratio with high body weight were recordered ($p \leq 0.05$) by OL. No mainly altered effects between both IO sites on most variables measured. However, there were significant influences among interactive treatments which related to *in ovo* injected substance in an injection site-dependent manner. It is concluded that improved hatchability, physiological and productive characteristics of heat-stressed chicks were achieved by IO of EG and OL.

Keywords: Antioxidant, Incubation, In ovo, Production, Stress

INTRODUCTION

The avian embryo develops its physiological activity and maintains homeostasis depending on closed system of limited nutritive content of egg. Before hatching time, several changes may occur to meet embryo energy that highly required to fulfill the hatching process, such as consumption of glycogen reserves, increase the gluconeogenesis and degradation of muscular protein (Givisiez *et al.,* 2020; Das *et al.,* 2021). These passive metabolic changes at the end of hatching may be handled by *in ovo* injection (IO) to supply conventional nutrients for embryo which their influences reflect on initial days and last up to few weeks post hatch (Coskun *et al*.,

2017; Al-Shammari *et al*., 2019). Nowadays, there are interesting works have been directed toward early delivering for phytochemicals extracted from potential plants to the growing embryos at different ages of incubation through application of IO. These bioactive compounds injected *in ovo* could mitigate the overall deleterious impacts during embryonic development through improving indigenous gut microflora (Hajati *et al.,* 2014), immune state and redox system (Khalifa *et al*., 2023) which affect hatchability (Akosile *et al.,* 2023a) and even modulating productive performance (Oke *et al.,* 2021) and meat quality (Wei *et al*., 2011) in post hatch time of chicks.

Heat stress is one of the most physiological causes for putative generation of oxidative stress in *vivo* by harmful increasing in levels of oxygen and nitrogen reactive species. During heat stress, cell injury and cellular mechanisms of apoptosis and necrosis may occur to confront the stress severity and activate the cellular adaptation against multiple stressful conditions (Shehata *et al.,* 2020). Thus, it was proved that heat stress negatively affects productive aspect, immune response, intestinal microbial balance, appetite hormones and favorable quality of meat and eggs in poultry (Shehata *et al.,* 2020; Goel 2021).

One of the effective strategies which have attracted much considerable attention to alleviate the heat stress problems in poultry production is using natural secondary metabolic substances in plants, such as polyphenols because of their powerful antioxidative influences (Hu *et al.,* 2019). Epigallocatechin-3-gallate (EG) is principle and abundant polyphenolic compound belongs to catechins groups existed in green tea (*Camellia sinensis*) leaves. EG has many pharmacological and therapeutic influences in veterinary practices because of its antioxidative, anti-inflammatory, antimicrobial, antihyperglycemic, antihypertensive, anti-allergic, hypocholesterolemic and immunostimulatory proprieties (Bartosikova and Necas, 2018). Interestingly, It was concluded that potent antioxidative activity of EG for inhibition of injurious free radicals is more than 25 and 100 times than vitamin E and vitamin C, respectively (Abd El-Hack *et al.,* 2020). Positive effects of EG supplemented in poultry diets regarding growth performance, modulating the antioxidant characteristics and other physiological aspects were previously confirmed during heat stress (Xue *et al.,* 2017; Luo *et al.* 2018; Song *et al*., 2019; Zhao *et al.,* 2021). Another major polyphenolic compound which is predominant in olive (*Olea europaea* L.) extract is oleuropein (OL). OL is characterized by its natural bitter taste and specific aroma which is included in list of secoiridoid group that produced by secondary metabolism in olive fruits and leaves (Acar-Tek and Ağagündüz, 2020). OL content in olive tissues is varied according to harvest season and method of extraction which is amounted 97 mg/g of olive leaf extract (Sarica and Toptas, 2014). OL is widely used in traditional medicinal and animal feeding as potent alternative to antibiotics because its exertion for many physiological influences, such as antioxidative, anti-inflammatory, hypolipidemic, hypoglycemic and antimicrobial activities as well as regulatory mechanism for blood pressure, cardiovascular failure and thyroid disorders (Durlu-Özkaya and Özkaya, 2011). Dietary importance for various concentrations of OL extract as natural antioxidant and productive promoter *in vivo* has been focused in many reports in poultry species under stressful or normal conditions (Sarıca *et al.* 2015; Oke *et al.,* 2017; Agah *et al.,* 2019; Shimao *et al.,* 2019).

Thermotolerance evaluation in newly hatched chicks based on IO of plant extracts is rare in published data and requires to be explored deeply. Therefore, the aim of the current research was to analyze influence of EG and OL as natural antioxidant extracts injected *in ovo* on the most important hatching parameters and subsequent physiological and productive characteristics of heat-stressed chicks during 24 hours post hatch and up to 6 weeks of age.

MATERIALS AND METHODS

Ethics of Experiment

The research was conducted in Poultry fa-

cility where belongs to Department of Animal Production Techniques, Al-Musaib Technical College, Al-Furat Al-Awsat Technical University. All guidelines issued by Ethical Committee in Department of Animal Production Techniques were followed regarding to animal welfare and management (No.22 of 1972).

Egg Incubation

The hatching eggs of Ross 308 strain were obtained from commercial hatchery source which originated from similar breeder flock at 40 weeks of age. All eggs were chosen individually based on their initial weight (58.12 g) and set in programmed incubator (Petersime Co., Belgium) until hatching time.

Application of *in Ovo* Injection and Experimental Groups

In total, 840 fertile eggs were candled and selected to perform procedure of IO on 12th day of embryonic development. IO was performed in two sites of egg determined by candling which involved air cell (AC) from blunt end of egg without contact embryonic membranes, and yolk sac (YS) which is close to equatorial axis of egg. Injection sites were precisely cleaned with cotton disinfected by 76% ethyl alcohol and injection was made by using 1 ml insulin syringe (25 gauge). After completing of IO, the injection sites were disinfected again and sealed with paraffin wax and all trays were moved back to the incubators as quickly as possible. EG and OL (97% purity, Naturalin Bio-Resources Co, Ltd) were both used as antioxidant extracts for IO. Aqueous solutions of these extracts were prepared by dissolving in distilled water depending on required dose for IO. The experimental eggs were randomly divided into 7 groups (n=120/ group) with 4 replications each. The groups were assigned to negative control group (NC) without IO and other 6 groups involved IO of 100 µl distilled water as positive control (PC) and 5 mg/100 µl each of EG and OL in both AC and YS sites of egg.

Hatched Chicks Management

After hatching, a total of 700 healthy chicks was selected (25 chicks per replicate) based on hatching rate in group. All chicks were reared in poultry house in floor pen equipped with the similar environmental conditions. Chicks were exposed to heat stress (40.0°C) at relative humidity (56%) for 24 hours post hatch immediately. The ambient temperature and relative humidity was recorded accurately using 5 temperature and humidity meters (Great Farm Co., Henan). At 2nd day, the remaining chicks after heat stress (n=420) were redistributed into 15 chicks/ replicate according to their initial groups previously. Thereafter, the temperature was adjusted to 34°C and gradually decreased until optimal level to 20°C at 42 day old of age. During whole rearing period, *ad libitum* feeding and continuous lighting were offered to birds. Birds were fed on balanced diets with crumble form in three age phases involving starter (1-10 days), grower (11-22 days) and finisher (23-42 days) according to NRC (1994).

Hatching Results and Chick Quality

After hatching, hatchability and mortality percentages in each replicate in group were determined by counting number of hatched chicks and dead embryos, respectively out of total number of eggs that were injected *in ovo*. Deformed chicks which are characterized by weakness and multiple deformities, such as malformed bones, twisted legs, absent eye, lack of upper beak were discarded and their percentages were calculated out of total number of hatched chicks. Body weight (BW) of hatched chicks was individually registered using a sensitive digital scale and relative BW in relation to initial egg weight was also registered. To determine chick length, 10 healthy chicks in replicate were laid done and stretched gently on its ventral part and length measurement was taken from the tip of the beak to the distal end of the middle toe using ruler. The value of eggshell conductance constant (K) as an indicator for egg weight loss was achieved by formula of Christensen *et al.* (2001).

Measurement of Body Temperature

To measure body temperature for heat-stressed chicks, 5 chicks were chosen from each replicate (20/ group) at 4 time intervals (06:00, 12:00, 18:00 and 24:00 hours). A digital thermometer (Beurer FT-09 ORAL, Germany) was used for this purpose by gentle inserting about 1 cm in rectum and the reading was recorded continuously in this position until it was stabilized by frequently alarm tone.

Productive Traits

The survivability in stressed chicks was registered from 1st till 2nd day post hatch. During entire experiment, BW and feed intake (FI) were taken and from these both values a feed conversion ratio (FCR) was obtained with taking into consideration total mortality. Moreover, production efficiency factor was also determined. At end of the experiment and after 10 hours fasting, 4 birds per replicate with the same average final BW in replicate were selected, weighed and slaughtered. The carcasses were eviscerated to measure relative weight of carcass yield in relation to live BW.

Serum Blood and Liver Sampling

On 2nd day, 5 chicks (1 male and 4 females) were chosen randomly in each replicate and were subjected to blood and liver sampling. The collected blood was harvested twice from the same bird, firstly from the brachial vein puncture and collection in K3-EDTA tubes for immediate counting of heterophil to lymphocyte (H/L) ratio. Secondly, blood was collected immediately after slaughtering from jugular vein and kept in serum separator gel tubes. Blood samples were transferred to laboratory, where tubes centrifuged at 1500 RPM for 15 minutes to separate and obtain serum which was placed at –20°C for conducting required analyses. From the same killed chicks, liver was carefully removed, collected and then snap-frozen in liquid nitrogen for redox status analyses. Liver tissue lysate was prepared by approximate homogenization of 0.3 g samples with ice-cold 0.9% sodium chloride buffer at 1 weight : 9 volume by using adjustable high speed homogenizer (MXBaoheng). After that, the homogenate was vortexed, shortly sonicated and centrifuged for 15 minutes at 4550×g and 4°C to obtain supernatant of lysate (Lu *et al.,* 2019). The supernatant was thereafter stored at −80°C until analysis time.

Oxidation Indicators

The total antioxidant capacity (TAC), antioxidative enzymes including glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) and oxidation biomarker, such as malondialdehyde (MDA), protein carbonyl (PCA) and heat shock protein 70 (HSP70) were analyzed in in serum and liver. The guidelines of various ready kits (Sigma Aldrich, St. Louis, MO, USA) was followed strictly and a spectrophotometer (Shenzhen, China) was used for this purpose. Each specific kit and its reagents characterized by their set of standards with calculated concentrations. TAC was measured using ferric reducing antioxidant power assay (Benzie and Strain, 1996). Regarding to GPx determination, the dithio nitro benzene method was followed. The SOD was estimated in accordance with xanthine oxidase method (Misra and Fridovich, 1972). CAT activity was measured depending on its amount in decomposition of H_2O_2 (Aebi, 1984). The analytical method of Salih *et al.* (1987) was carried out to determine MDA value using thiobarbituric acid reactive substance concentration method. PCA as indicator of protein oxidation was evaluated based on slightly modified protocol of Levine *et al.* (1990) by using the reactive compound,2,4 dinitrophenylhydrazine as a reagent. Moreover, HSP70 was detected based on manufacturer's instructions mentioned in enzyme-linked immunosorbent assay (ELISA) ready kit (Shanghai Sunred Biological Technology) which is designed to chicken HSP70 and using microplate reader (BK-EL10C, Biobase, USA).

Biochemical Markers

The spectrophotometer was used to investigate all biochemical parameters in serum by following routinely diagnostic steps for determination of glucose by Cromatest kit (Spanish) and

using kits (Biolabo, French) for cholesterol, total protein (Young, 2000), creatinine and uric acid (Burtis and Ashwood, 1999) measurements. Moreover, a method of Reitman and Frankel (1957) which stated in bioassay kit (Randox, English) was applied to measure the enzymatic activity of both aspartate transaminase (AST) and alanine aminotransferase (ALT).

Hormonal Analysis and H/L Ratio Measurement

In serum samples, accredited chicken ELISA kits (MyBioSource, Inc., USA) were used to analyze triiodothyronine (T3), thyroxine (T4) and thyroid-stimulating hormone (TSH) whereas a standard radioimmunoassay kit (IDS, Boldon, UK) was used for measurement of corticosterone level. Detailed steps of each analytical step in kit was followed according to its instructions. The absorbance was then determined at defined value in reference wavelength by ELISA microplate reader. The H/L ratio was calculated from anticoagulated blood tubes by following method of Burton and Guion (1968).

Data Analysis

The data were set to ANOVA and analyzed by the general linear model according to the completely randomized design through software of SAS (2012) to find the main effect of *in ovo* injection of antioxidant factor (NC, PC, EG and OL), the main effect of *in ovo* injection site factor (AC and YS) and interactive treatments (4×2). Duncan's multiple range test (Duncan, 1955) was applied to determine the significant differences among experimental groups. The level of $p \leq 0.05$ was used to consider the significance according to the following statistical model:

$$Y_{ijk} = \mu + IOA_i + IOS_j + (IOA \times IOS)_{ij} + e_{ijk}$$

where Y_{ijk}, observation; μ, overall mean; IOA_i, effect of *in ovo* injection of antioxidant; IOS_j, effect of *in ovo* injection site; $(IOA \times IOS)_{ij}$, interaction between *in ovo* injection of antioxidant and *in ovo* injection site, and e_{ij}, random error.

RESULTS AND DISCUSSION

Hatchability and Chicks Parameters

Table 1 shows that the best hatchability and the worst mortality ($p \leq 0.05$) were achieved by

Table 1. Effect of *in ovo* injection of epigallocatechin-3 gallate and oleuropein on hatching results of heat-stressed chicks

Groups	Traits						
	Hatchability (%)	Mortality (%)	Deformed chicks (%)	Chick weight (g)	Chick weight (%)	Chick length (mm)	K
	In ovo injection of antioxidant (IOA)						
NC	90.83^{b}	9.17^{a}	3.67^{a}	42.34^{ab}	72.85^{bc}	174.20^{bc}	5.77^{ab}
PC	90.84^{b}	9.16^{a}	0.00^{b}	41.81^{b}	71.94^{c}	171.79^{d}	5.90^{a}
EG	94.59^{a}	5.42^{b}	0.45^{b}	43.57^{a}	74.97^{a}	176.43^{a}	5.23^{c}
OL	93.33^{a}	6.67^{b}	0.00^{b}	43.17^{a}	74.27^{ab}	173.52^{cd}	5.38^{bc}
	In ovo injection site (IOS)						
AC	92.10	7.92	1.14	42.62	73.33	173.14b	5.58
YS	92.71	7.29	0.92	42.83	73.69	174.83^{a}	5.49
Pooled SEM	9.23	1.14	0.00	6.41	10.51	4.36	1.51
	P-value						
IOA	0.018	0.023	0.043	0.037	0.044	0.019	0.045
IOS	0.101	0.098	0.110	0.088	0.212	0.045	0.124
IOA × IOS	0.025	0.032	0.041	0.031	0.023	0.017	0.026

NC: without *in ovo* injection, PC: *in ovo* injection of 100 µl distilled water/egg, EG and OL: *in ovo* injection of 5 mg each of epigallocatechin-3 gallate and oleuropein/egg, respectively, AC and YS: *in ovo* injection in air cell and yolk sac, respectively. K: eggshell conductance constant, SEM: standard error mean, a,b,c: means within same columns with different superscripts differ significantly at $p \leq 0.05$.

EG and OL with low relative deformed chicks ($p \leq 0.05$) were obvious in all IOA groups compared with NC. All IOA groups did not differ significantly from NC regarding the absolute chick weight. In comparison to NC, an increased relative weight of chick and its length with lowering ($p \leq 0.05$) K value was found in EG. Depending on IOS, it is obvious that chick length was increased ($p \leq 0.05$) by YS compared with AC. Also, significant differences ($p \leq 0.05$) in these parameters were present among all interactive treatments (IOA×IOS). High hatchability with low mortality in phenolic compounds groups (EG and OL) injected *in ovo* is probably due to the beneficial effects of IO of these extracts to provide required energy and combat oxidative stress during hatching time (Givisiez *et al.,* 2020). This might explain the importance of quick absorption for EG and OL during embryogenesis and easily transfer to embryo by blood vessels of YS that is in direct contact with developing embryo. Also, it is not surprisingly that IO of substances through AC can pass generally to vascular system of several embryonic membranes surrounding the embryo and appear their efficient influence (Das *et al.,* 2021). These changes were obvious to make significant differences between the interactive treatments, but not on the base of IOS factor. An absent information on the direct influence of IO of EG and OL extracts was documented, although their dietary effect was illustrated in literature. Inconsistent result was obtained recently by Khalifa *et al*. (2023), that IO into YS at 14 days of incubation with 1000 ppm olive pulp extract did not change hatchability and BW whereas the dose of 500 ppm decreased the hatchability.

The decreasing in deformed chicks ratio in IOA groups and high chick length in EG may be related to bioavailability of EG in embryonic tissues which lowers the risk of osteoporotic occurrence, apoptotic osteoblasts and modulates the inflammatory response against bone fractures and abnormalities by different mechanisms (Bartosikova and Necas, 2018). Likewise, OL is responsible for therapeutic importance against infections by its antibacterial, anti-fungal, anti-parasitic, anti-yeast and antiviral effects (Durlu-Özkaya and Özkaya, 2011). In accordance with Ranjbar *et al.* (2019), IO through amniotic sac during incubation at 14th and 17.5th day with 15 or 30 mg of naringin enhanced skeletal system development and bone quality of hatched chicks. Also, chick length at hatch was found to be increased through IO with 3 mg of black cumin into AC at 17.5th day of incubation (Peşmen, 2022). High relative weight and length of chick in EG and YS groups is perhaps correlated with low K value. It is speculated that high K value refers to amount of moisture loss from egg which is undesirable event for embryonic development that depends on weights of initial egg and hatched chick and length of incubation time (Christensen *et al.,* 2001). Differently, Khaligh *et al.* (2017) concluded that feeding chicken embryos at 18 day of age via amniotic cavity with 4.5 mg quercetin or chrysin did not affect the relative BW of chicks. Similar reports to us referred that absolute BW of chick was not influenced by IO of plant extract solutions, such as equol (20 and 100 mg) at 7 days of embryonic age into albumen (Wei *et al*., 2011) or different levels (3, 4.5 and 6 mg) of grape seed extract at 18 days of incubation into AC (Hajati *et al.,* 2014).

Body Temperature and Antioxidant Activity

Table 2 describes the effect of groups on rectal temperature measured during 24 hours post hatch. At 6:00 hours, this value was lower ($p \leq 0.05$) in EG and OL than NC. However, no differences in this value among groups at 12:00 and 18:00 hours were achieved. OL reduced a rectal temperature ($p \leq 0.05$) compared with NC at 24:00 hours and in overall mean. Also, YS decreased this value only at 6:00 hours compared to AC. The differences ($p \leq 0.05$) among interactive treatments were only at 6:00, 24:00 hours and in overall mean. Compared with NC in Table 3, enhanced levels ($p \leq 0.05$) of TAC and CAT in serum were achieved by OL. Significant increase in SOD and decrease in MDA value was registered by EG and OL. PCA value was decreased ($p \leq 0.05$) by EG whereas all IOA groups

Table 2. Effect of *in ovo* injection by epigallocatechin-3 gallate and oleuropein on rectal temperature (℃) for 24 hours post hatch of heat-stressed chicks

Groups	Traits				
	6:00 hours	12:00 hours	18:00 hours	24:00 hours	Overall mean
In ovo injection of antioxidant (IOA)					
NC	41.36[a]	41.47	41.46	41.76[a]	41.51[a]
PC	41.42[a]	41.29	41.39	41.49[a]	41.40[a]
EG	40.28[c]	41.13	41.13	41.33[ab]	40.97[ab]
OL	40.66[bc]	41.08	41.29	40.76[b]	40.94[b]
In ovo injection site (IOS)					
AC	41.07[a]	41.25	41.39	41.49	41.30
YS	40.79[b]	41.23	41.23	41.17	41.10
Pooled SEM	2.17	1.54	1.29	1.73	1.60
P-value					
IOA	0.044	0.140	0.201	0.049	0.035
IOS	0.033	0.099	0.093	0.113	0.078
IOA × IOS	0.037	0.080	0.076	0.044	0.029

NC: without *in ovo* injection, PC: *in ovo* injection of 100 µl distilled water/egg, EG and OL: *in ovo* injection of 5 mg each of epigallocatechin-3 gallate and oleuropein/egg, respectively, AC and YS: *in ovo* injection in air cell and yolk sac, respectively. SEM: standard error mean, a,b,c: means within same columns with different superscripts differ significantly at p≤0.05.

Table 3. Effect of *in ovo* injection of epigallocatechin-3 gallate and oleuropein on serum antioxidative indicators of heat-stressed chicks

Groups	Traits						
	TAC (U/ml)	GPx (U/L)	SOD (U/ml)	CAT (U/ml)	MDA (µmol/L)	PCA (nmol/mg protein)	HSP70 (pg/ml)
In ovo injection of antioxidant (IOA)							
NC	5.24[b]	23.43	78.54[cd]	2.92[bc]	9.64[ab]	4.72[a]	200.23[a]
PC	5.46[b]	23.23	77.09[d]	2.54[c]	9.72[a]	4.79[a]	189.51[b]
EG	6.20[ab]	24.36	82.24[b]	3.60[ab]	8.79[b]	3.39[b]	171.57[c]
OL	7.62[a]	24.89	92.75[a]	4.62[a]	8.53[b]	3.75[ab]	165.63[d]
In ovo injection site (IOS)							
AC	6.13	23.66	83.67[a]	3.14	9.21	4.09	180.91[b]
YS	6.14	24.29	81.64[b]	3.71	9.18	4.24	182.56[a]
Pooled SEM	1.50	5.62	6.87	0.98	2.01	1.11	52.14
P-value							
IOA	0.047	0.321	0.043	0.038	0.049	0.037	0.026
IOS	0.231	0.111	0.041	0.243	0.381	0.099	0.043
IOA × IOS	0.042	0.039	0.021	0.048	0.027	0.035	0.049

NC: without *in ovo* injection, PC: *in ovo* injection of 100 µl distilled water/egg, EG and OL: *in ovo* injection of 5 mg each of epigallocatechin-3 gallate and oleuropein/egg, respectively, AC and YS: *in ovo* injection in air cell and yolk sac, respectively. TAC: total antioxidant capacity, GPx: glutathione peroxidase, SOD: superoxide dismutase, CAT: catalase, MDA: malondialdehyde, PCA: protein carbonyl, HSP70: heat shock protein 70, SEM: standard error mean, a,b,c,d: means within same columns with different superscripts differ significantly at p≤0.05.

reduced HSP70 compared with NC. High SOD and low HSP70 values (p≤0.05) were registered by AC compared to YS**.**

In Table 4, it was obvious that EG and OL recorded high values (p≤0.05) in liver TAC, GPx and SOD compared to controls. Low MDA content (p≤0.05) was for OL compared to others. No significant differences were confirmed between IOS groups with respect to these parameters. The interactive treatments were different among them (p≤0.05) in antioxidant status of both serum and liver. The polyphenolic compounds can scavenge excessive free radicals that lead to mitochondrial dysfunction, downregulate the gene expression of stress-related proteins and maintain the balance of redox in body during heat stress (Hu *et*

al., 2019). This would explain the hypothermic effect of EG and OL extracts delivered to embryos in lowering body temperature and providing resistance against heat stress accompanied with overall improving the antioxidant system and decreasing indicators of oxidation levels in serum and liver.

The antioxidive protection for 300 and 600 mg EG/kg diet under heat stress in broiler was proved earlier by Xue *et al.* (2017) and Luo *et al.* (2018) via increasing GPX, CAT and SOD enzymes and decreasing MDA content in serum and liver. Also, dietary EG can enhance expression of antioxidant-related proteins, such as nuclear factor erythroid 2-related factor (Nrf2), sirtuin 1, peroxisome proliferator-activated receptor-γ coactivator-1 alpha (PGC-1α) and phospho-AMP- activated protein kinase (p-AMPKα) and modulation of AMPK/ sirtuin 1 pathway in liver of heat-stressed broilers (Xue *et al.,* 2017). Moreover, in heat-stressed quails, Orhan *et al.* (2013) suggested that EG delivered in diet (200 or 400 mg/kg) suppressed at greater extent the stress-related gene expression and inflammatory markers of gene transcription in liver which involving 60, 70 and 90 HSP, cyclooxygenase-2 and activator protein-1 components (c-Jun and c-Fos). Identical result referred that antioxidative status of hatched chicks was enhanced at 5 weeks through feeding embryos by 1000 mg olive pulp which resulted in high catalase, SOD and TAC in muscles (Khalifa *et al.,* 2023). The improvement in oxidant/antioxidant balance might be related to positive influence of OL to reduce body temperature of broilers reared in hot climate (Oke *et al.,* 2017). Likewise, the antioxidant activity of 0.5 ppm OL offered in diet was confirmed to stimulate the key antioxidant gene expression of PGC-1α, avian uncoupling protein, sirtuin-1 and -3 and MnSOD with inhibiting of carbonyl content and oxidative damage in mitochondria of broiler muscle (Shimao *et al.,* 2019). Although the differences between AC and YS groups were mostly insignificant in lowering body temperature and improving redox status in serum and liver, the significance of the interac-

Table 4. Effect of *in ovo* injection of epigallocatechin-3 gallate and oleuropein on antioxidative indicators in liver of heat-stressed chicks

Groups	Traits					
	TAC (U/mg protein)	GPx (U/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	MDA (μmol/mg protein)	PCA (nmol/mg protein)
In ovo injection of antioxidant (IOA)						
NC	1.04^{ab}	15.43^{b}	62.54^{b}	2.12	1.16^{a}	6.42
PC	0.70b	15.64^{b}	62.18^{b}	2.16	0.79^{a}	6.92
EG	1.64^{a}	18.07^{a}	71.62^{a}	2.37	0.68^{ab}	6.42
OL	1.71^{a}	18.13^{a}	69.80^{a}	2.59	0.64^{b}	6.59
In ovo injection site (IOS)						
AC	1.24	17.15	66.43	2.21	0.82	6.58
YS	1.31	16.49	66.64	2.41	0.81	6.59
Pooled SEM	0.07	4.99	7.54	0.27	0.02	1.12
P-value						
IOA	0.042	0.035	0.046	0.176	0.028	0.231
IOS	0.231	0.099	0.412	0.112	0.254	0.312
IOA × IOS	0.023	0.038	0.043	0.032	0.019	0.081

NC: without *in ovo* injection, PC: *in ovo* injection of 100 µl distilled water/egg, EG and OL: *in ovo* injection of 5 mg each of epigallocatechin-3 gallate and oleuropein/egg, respectively, AC and YS: *in ovo* injection in air cell and yolk sac, respectively. TAC: total antioxidant capacity, GPx: glutathione peroxidase, SOD: superoxide dismutase, CAT: catalase, MDA: malondialdehyde, PCA: protein carbonyl, SEM: standard error mean, a,b: means within same columns with different superscripts differ significantly at $p \leq 0.05$.

tions were more prominent in improving these variables, and perhaps it might be due to the effect of IOA factor.

Biochemical Parameters

In Table 5, serum protein was increased by OL compared to PC. EG and OL caused to decrease ($p \leq 0.05$) glucose, cholesterol and AST. There were no significant differences among IOA groups with respect to serum uric acid, creatinine and ALT. Depending on IOS, AC lowered glucose value compared to YS. Mostly, the differences ($p \leq 0.05$) were achieved among interactive treatments. There is no question that blood biochemistry is associated with antioxidant capacity. Regardless of IOS factor, these positive changing or stability of some metabolite values in serum of EG and OL and differences among IOA×IOS groups might reflect any alteration in metabolic pathways of protein, carbohydrate and fat in liver of broiler under stress (Xue *et al.,* 2017).

Liver which is the main organ for metabolism, it was reported that its inflammatory, antioxidant and detoxifying mechanisms were enhanced under feeding EG via upregulating Keap1/Nrf2 signaling that resulted in attenuating excessive apoptosis in hepatocyte mitochondria and mitigating adverse effect of pathological damage in liver (Wang *et al.,* 2022). Therefore, these measured indicators might be a reasonable explanation to alleviate stress extent. In accordance, Tuzcu *et al.* (2008) noted that was considerable lowering in serum cholesterol and glucose of stressed quails fed diet containing 200 or 400 mg of EG/kg for 42 days of age. Also, Luo *et al.* (2018) observed that supplementing diet for heat-stressed chickens by EG exhibited a linear increase in content of serum protein and decrease in levels of glucose, cholesterol and AST activity at 35 days of age. In addition, a low cholesterol level in serum of heat-stressed chickens was achieved during supplementing 200 or 400 mg of olive extract/kg of diet from 28 to 42 days of age (Agah *et al.,* 2019). Also, consistent data indicated that feeding heat-stressed quails with 200 ppm olive leaf extract containing 103 mg of OL/g had no impact on serum uric acid and creatinine at 43 -day-old (Erişir *et al.,* 2020).

H/L ratio and hormonal response

Table 6 declares that decreased H/L ratio ($p \leq 0.05$) was noticeable in OL. Levels of corticosterone and T4 were decreased and increased ($p \leq 0.05$) respectively by both EG and OL. In the meanwhile, T3 and TSH values were not significantly changed by IOA groups. Increased value of T4 was obtained by YS compared to AC with

Table 5. Effect of *in ovo* injection of epigallocatechin-3 gallate and oleuropein on serum biochemistry of heat-stressed chicks

Groups	Traits						
	Protein (g/dl)	Glucose (mg/dl)	Cholesterol (mg/dl)	Uric acid (mg/dl)	Creatinine (mg/dl)	ALT (U/L)	AST (U/L)
In ovo injection of antioxidant (IOA)							
NC	2.42^{ab}	127.98^{a}	100.33^{a}	4.15	0.52	4.43	119.54^{a}
PC	2.19^{b}	126.09^{ab}	99.11^{a}	3.86	0.48	4.02	119.73^{a}
EG	2.42^{ab}	125.93^{b}	96.75^{b}	3.94	0.40	4.01	116.29^{b}
OL	2.80^{a}	121.98^{c}	94.29^{c}	3.28	0.37	3.79	116.99^{b}
In ovo injection site (IOS)							
AC	2.41	124.51^{b}	98.11	3.62	0.45	3.93	118.49
YS	2.48	126.48^{a}	97.13	3.99	0.43	4.19	117.78
Pooled SEM	0.95	39.34	15.66	0.99	0.02	0.13	36.44
P-value							
IOA	0.043	0.032	0.027	0.099	0.114	0.216	0.041
IOS	0.231	0.047	0.232	0.211	0.311	0.107	0.086
IOA × IOS	0.024	0.039	0.041	0.083	0.100	0.096	0.038

NC: without *in ovo* injection, PC: *in ovo* injection of 100 µl distilled water/egg, EG and OL: *in ovo* injection of 5 mg each of epigallocatechin-3 gallate and oleuropein/egg, respectively, AC and YS: *in ovo* injection in air cell and yolk sac, respectively. ALT: alanine aminotransferase, AST: aspartate aminotransferase, SEM: standard error mean, a,b,c: means within same columns with different superscripts differ significantly at $p \leq 0.05$.

Table 6. Effect of *in ovo* injection of epigallocatechin-3 gallate and oleuropein on H/L ratio and serum hormones of heat-stressed chicks

Groups	Traits				
	H/L ratio	Corticosterone (ng/ml)	T3 (ng/ml)	T4 (ng/ml)	TSH (ng/ml)
	In ovo injection of antioxidant (IOA)				
NC	0.45[a]	10.47[a]	1.01	66.32[cd]	0.07
PC	0.43[a]	10.65[a]	1.14	64.32[d]	0.08
EG	0.41[ab]	7.99c	1.18	76.42[b]	0.09
OL	0.35[b]	9.09[bc]	1.31	87.59[a]	0.08
	In ovo injection site (IOS)				
AC	0.40	9.69	1.13	71.34[b]	0.07
YS	0.41	9.41	1.19	75.98[a]	0.08
Pooled SEM	0.00	2.57	0.11	10.43	0.00
	P-value				
IOA	0.022	0.034	0.181	0.036	0.219
IOS	0.098	0.154	0.243	0.039	0.362
IOA × IOS	0.030	0.029	0.099	0.041	0.131

NC: without *in ovo* injection, PC: *in ovo* injection of 100 µl distilled water/egg, EG and OL: *in ovo* injection of 5 mg each of epigallocatechin-3 gallate and oleuropein/egg, respectively, AC and YS: *in ovo* injection in air cell and yolk sac, respectively. H/L ratio: heterophil to lymphocyte ratio, T3: triiodothyronine, T4: thyroxine, TSH: thyroid-stimulating hormone, SEM: standard error mean, a,b,c,d: means within same columns with different superscripts differ significantly at p≤0.05.

Table 7. Effect of *in ovo* injection of epigallocatechin-3 gallate and oleuropein on productive performance of heat-stressed chicks

Groups	Traits						
	SURV (%)	BW (g)	FI (g)	FCR	Mortality (%)	CY (%)	PEF
	1–2 day	6 week	1–6 week	1–6 week	1–6 week	6 week	6 week
	In ovo injection of antioxidant (IOA)						
NC	81.00[b]	2455.34[bc]	3876.23[a]	1.61[a]	6.67[a]	74.08	339.68[c]
PC	80.21[b]	2445.20[c]	3794.17[ab]	1.57[ab]	5.83[a]	74.24	347.31[bc]
EG	90.16[a]	2598.00[ab]	3759.37[b]	1.46[b]	3.34[b]	75.19	407.61[ab]
OL	91.63[a]	2619.35[a]	3822.33[a]	1.48[b]	0.84[c]	75.16	416.46[a]
	In ovo injection site (IOS)						
AC	84.06b	2514.58	3823.23	1.54	5.00[a]	74.56	368.41[b]
YS	87.44a	2544.37	3802.81	1.52	3.34[b]	74.78	387.12[a]
Pooled SEM	12.76	65.87	23.19	0.12	0.94	8.77	52.54
	P-value						
IOA	0.042	0.049	0.036	0.028	0.038	0.312	0.041
IOS	0.039	0.154	0.098	0.231	0.037	0.229	0.033
IOA × IOS	0.033	0.035	0.022	0.032	0.042	0.028	0.026

NC: without *in ovo* injection, PC: *in ovo* injection of 100 µl distilled water/egg, EG and OL: *in ovo* injection of 5 mg each of epigallocatechin-3 gallate and oleuropein/egg, respectively, AC and YS: *in ovo* injection in air cell and yolk sac, respectively. SUR: survivability, BW: body weight, FI: feed intake, FCR: feed conversion ratio, CY: carcass yield, PEF: production efficiency factor, SEM: standard error mean. a,b,c: means within same columns with different superscripts differ significantly at p≤0.05.

no significant differences between AC and YS in other traits. Moreover, interactive treatments differed (p≤0.05) among them in most of these parameters. OL considerably enhanced immune response and boosted cellular immunity by decreasing H/L ratio. This result was in harmony with the observation of Sarıca *et al.* (2015), that was decreasing in H/L ratio in heat-stressed quail

at 35 days fed 200 mg OL/kg diet. Different data was based on Oke *et al.* (2017), that was no effect of 10 and 15 ml of olive leaf extract containing OL at 4.4 mg/ml offered per liter of drinking water on H/L ratio of broilers reared in hot dry season till 8 weeks. Lowering in corticosterone level for IO and EG groups gives an indicator to supply resilience against stress and might broadly be linked with improved anitoxidant status previously. It was reported that corticosterone level in plasma expressed by catabolic influence on functional proteins and its degradation in skeletal muscle of broilers was not changed by feeding 0.1, 0.5 and 2.5 ppm OL in diet (Shimao *et al.,* 2019).

High value of T4 achieved by IO of EG and OL especially under main effect of YS injection might reflect the positive effect of IO. T4 is considered as one of principle hormones in avian embryo and chicks that converted into its active form (T3). Both T3 and T4 are produced under control of TSH and are important in regulating metabolism, muscular growth, heat production, eggshell pipping during hatching and mobilization of glycogen and fat stores (Givisiez *et al.,* 2020). Comparable results indicated that levels of T4 along with T3 in blood were enhanced in hatched chicks by IO of 1000 mg olive pulp extract (Khalifa *et al.,* 2023) or 30 mg naringin (Ranjbar *et al.,* 2019). Opposite data to us, it was confirmed that high amount of serum T3 in hatched chicks was registered by IO into AC site at 17.5 days of heat-stressed embryos with 6 mg of black cumin (Oke *et al.,* 2021) or with 2 mg of clove (Akosile *et al.,* 2023b). It is reasonable that present significances among IOA × IOS resulted from influence of IOA factor rather than influence of IOS factor.

Productive Performance

Table 7 shows that was superiority ($p \leq 0.05$) in SUR, BW, FCR and PEF with inferior mortality ($p \leq 0.05$) in EG and OL and decreased FI in EG compared with NC. No significant differences among all IOA groups in CY. Low mortality and high PEF were in favor of YS compared to AC. Additionally, the differences ($p \leq 0.05$) were present in IOA × IOS treatments regarding all productive traits. It is intriguing there was crucial influence of IO in enhancing subsequent productive performance especially under effect of IOA factor which led to significance effects among interactive groups. IO of powerful antioxidants might lead to stimulate absorption and utilize yolk materials efficiently by growing stressed chick (Akosile *et al.,* 2023a) which was the basic reason behind improving overall performance. YS contains crucial substances that nutritionally and immunologically support embryo and hatched chicks because of its content of glycogen stores, proteins, lipids and maternal antibodies (Givisiez *et al.,* 2020). These alterations can be translated into reinforcing physiological parameters with reduction of body temperature based on IO. Moreover, all improvements are presumably associated with antioxidative and anti-inflammatory protection of EG in digestive mucosa of intestine which result in activating intestinal absorption under stress status of broilers (Song *et al.,* 2019). Besides, OL is characterized by its fast ingestion by intestine in direct relationship with its dose (Shimao *et al*., 2019) and thus plays an obligate role in improving the digestibility of nutrients in ileum section of stressed poultry (Agah *et al.,* 2019).

In general, microbial balance and composition, immune system and health of gut contents are physiologically stimulated by IO of antioxidant compounds for growing embryos which might reflect on productivity post hatch (Das *et al.,* 2021). Thus, Coskun *et al.* (2017) opined that IO of 0.2 ml of pollen extract into AC at 18th day of incubation was beneficial to increase length of ileal villi and stimulate growth of microbial population in caeca by increasing numeration of *Saccharomyces cerevisiae*, lactic acid bacteria with lowering of Enterobactericaea and yeast at 21 day of broiler exposed to fasting stress for 24 or 48 hours post hatch. Compatible results reported the importance of plant extracts to modulate thermal challenge and boosting productive aspects in stressed poultry. As noted by Oke *et al.* (2021), that were high BW and low FCR at 56 d days for broilers derived from IO of

6 mg of black cumin during exposure to thermal stress in embryonic phase and 1 hour before marketing. More recently, it was concluded that IO of 2 and 4 mg of clove and cinnamon could alleviate body temperature and improve FCR by increasing BW gain and decreasing FI at 56 days of broilers (Akosile *et al.,* 2023b). Contradictory data proved that feeding embryos on day 17.5 of incubation with 0.01, 0.03 and 0.05 ml of nanocurcumin extract through amniotic cavity did not show any effects on FCR and BW but decreased FI in broilers reared under heat stress for 24 days (Heidary *et al*., 2020).

CONCLUSION

The results demonstrated that *in ovo* injection at 12 days of embryogenesis with either 5 mg EG or OL improved hatching parameters and eliminated adverse effect of short heat stress in chicks post hatch. These positive effects led to boosting final productive performance by enhanced antioxidant capacity, physiological values and hormonal response of heat-stressed chicks. Very low effect between *in ovo* injection sites (AC and YS) on variable studied was obvious. However, the interaction effects among treatments depending on *in ovo* injected antioxidant and *in ovo* injection site were achieved significantly.

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