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Effects of in ovo injection of chrysin, quercetin and ascorbic acid on hatchability, somatic attributes, hepatic oxidative status and early post-hatch performance of broiler chicks

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Summary

An experiment was conducted to evaluate the effects of in ovo injection of chrysin, quercetin and ascorbic acid on hatchability, somatic attributes, hepatic antioxidant status and early post-hatch growth performance of broiler chicks. Four hundred and eighty embryonated broiler breeder eggs containing live 18-day-old embryos were divided into six groups of 80 eggs each. One group remained intact and served as a control group (i), whereas the other five groups were injected with the prepared injection solutions as follows: (ii) 0.05 ml distilled water; (iii) 0.05 ml distilled water containing 6 mg ascorbic acid; (iv) 0.05 ml dimethyl sulfoxide (DMSO); (v) 0.05 ml DMSO containing 4.5 mg quercetin; and (vi) 0.05 ml DMSO containing 4.5 mg chrysin. The hatchability rate, hatching weight, residual yolk sac weight, yolk sac-free body weight, liver weight, hepatic glutathione peroxidase and total superoxide dismutase activities, as well as malondialdehyde concentrations, were not affected by the injected solutions. There were no differences between chicks hatched from the control and in ovo injected eggs in weight gain, feed intake and feed conversion ratio from 0 to 11 days of age. However, the specific contrast performed between the in ovo injected groups and intact eggs revealed that in ovo injection significantly increased hatchability rate (p = .0493). This finding also implies that our injection procedure was harmless. In conclusion, the intra-egg injection of chrysin, quercetin or ascorbic acid at the injection rates used in this study did not have a significant effect on hatchability, somatic characteristics, early growth performance and hepatic antioxidant status of broiler chicks. However, the overall hatchability was higher in the in ovo injected eggs as compared to non-injected ones. These findings also confirmed the harmlessness of the procedure developed for in ovo injection in this study.

KEYWORDS
ascorbic acid, broiler breeder eggs, chrysin, in ovo injection, quercetin

1 | INTRODUCTION

Unlike mammals, birds have a limited source of nutrients for embryonic development reserved in the egg through some specialised maternal mechanisms (Uni, Yadgary, & Yair, 2012). Under certain conditions, especially in modern broiler chickens, this nutritious pack may not be sufficient to fulfil the embryo requirements resulting in poor embryo development, and consequently reduced hatchability and chick quality. The imbalance between the egg nutrient content and embryo requirements partially may be prevented by the provision of additional and highly bioavailable sources of essential nutrients in maternal diets (Favero et al., 2013; Urso et al., 2015). Recently, with the advent of

in ovo injection technology, poultry researchers found an opportunity to realise the dream of post-laying fortification of hatching eggs with exogenous nutrients (Kadam, Barekatain, K-Bhanja, & Iji, 2013).

Antioxidants are substances that protect cells from the oxidative damages caused by unstable molecules known as free radicals. They are necessary for embryo health and survivability, particularly during the last 3 days of incubation (Malheiros, Ferket, & Gonçalves, 2012).

During the time of hatch, broiler chick embryos show an increased susceptibility to oxidative stress probably due to heightened metabolic rate and O_2 consumption, as well as high levels of tissue polyunsaturated fatty acids and insufficient natural antioxidant reserves (Malheiros et al., 2012). The embryo uses a variety of enzymatic (i.e., superoxide dismutase, glutathione peroxidase, catalase) and non-enzymatic (vitamin E, ascorbic acid, carotenoids, reduced glutathione, coenzyme Q, etc.) antioxidants as well as mineral cofactors (Se, Zn, Mn and Fe) to combat the oxidative stress. Among these components, vitamin E, carotenoids and cofactor minerals are driven from the maternal diet, and the others are synthesised in the tissues (Surai & Fisinin, 2012).

Vitamin C or ascorbic acid (ASA) is a physiologically multifunctional compound needed for a variety of processes in the animal body, including collagen synthesis, lipid metabolism, absorption of inorganic iron, immune system function, protection against free radical damages and reduction of oxidised vitamin E to its active form (Bender, 2003). Studies involving the measuring ASA content of hatching eggs have shown that fresh unincubated eggs are devoid of ASA (Rinaldini, 1960; Surai, Noble, & Speake, 1996). Rinaldini (1960) reported that the absolute amount of vitamin C in embryo body increases gradually after incubation, whereas its concentration (microgram per mg of body weight) showed a downward trend with the most marked falls observed in brain and muscle. It has been reported that hatchling somatic characteristics, antioxidant status and post-hatch performance may be improved by in ovo delivery of exogenous antioxidants, including ascorbic acid (Hajati, Hassanabadi, Golian, NassiriMoghaddam, & Nassiri, 2014; Lee et al., 2014).

The term "flavonoid" refers to a wide range of polyphenols sharing a common skeleton of phenylchroman (Vassallo, 2008). These compounds, the majority of them showing antioxidant properties, are divided into seven major subclasses, including flavonols, flavones, flavanones, flavanols, anthocyanins, isoflavones and proanthocyanidins (Murphy et al., 2003). Several mechanisms have been proposed for antioxidant activity of flavonoids, including direct scavenging of free radicals, nitric oxide and peroxynitrite, inhibition of xanthine oxidase activity and interaction with enzymatic antioxidant systems (Nijveldt et al., 2001). Quercetin, the most abundant (Ebadi, 2007) and biologically active (Vassallo, 2008) flavonoid belonging to flavonols, is found in vegetable foods and fruits. Chrysin, another example of flavonoids belonging to the flavone subclass, is naturally present in many plant extracts, honey, and propolis (Rapta, Misík, Stasko, & Vrábel, 1995; Williams, Harborne, Newman, Greenham, & Eagles, 1997). Both chrysin and quercetin exhibit marked antioxidant properties (Anand, Mohamed Jaabir, Thomas, & Geraldine, 2012; Liu et al., 2014). Supplemental quercetin at a level of 1500 mg/kg of diet caused significant reductions in splenic heat shock protein 70 expression as well as in splenic and cecal *Salmonella enteritidis* population in heat stressed broiler chickens (Soleimani, Zulkifli, Hair-Bejo, Omar, & Raha, 2012). Enzymatic and non-enzymatic antioxidants increased significantly, and D-galactose-induced oxidative damages were reduced in rats receiving chrysin at 20 mg/kg body weight in a study by Anand et al. (2012).

Previously published studies involving intra-egg delivering plant-derived antioxidant compounds have mostly used crude extracts (Hajati et al., 2014) or other plant-derived complex products (Moghaddam, Karimi, Borji, Bahadori, & Abdolmohammadi, 2013). Hajati et al. (2014) reported that in ovo injection of grape seed extract at a rate of 4.5 mg per egg improved hatchability rate, glutathione peroxidase (GPX) activity and early post-hatch performance of broiler chicks. The authors stated that the beneficial effects of grape seed extract may be attributed to the flavonoids existing in the product.

To our knowledge, there are no published data on the effect of in ovo injection of pure flavonoids on broiler chick embryos/hatchlings. Therefore, this study aimed to evaluate the effects of in ovo injection of chrysin, quercetin and/or ascorbic acid on hatchability rate, somatic attributes, hepatic oxidative status and early post-hatch performance of broiler chicks.

2 | MATERIALS AND METHODS

2.1 | Ethical approval

The experimental procedure was approved by the Animal care and use committee of the Ferdowsi University of Mashhad, Iran.

2.2 | Developing the injection procedure

A preliminary study was conducted with 200 embryonated broiler breeder eggs (embryonic day 18) to optimise the injector needle length and to determine the appropriate site of injection on eggshell to achieve an acceptable delivery into the amniotic cavity. The eggs were individually exposed to the light emitted from the candling box window in a way that their lateral bulge abutted the edge of lighting window with the blunt end held upward. Then the egg was turned around its longitudinal axis until the nearest point of the embryonic shadow to the air sac appeared, and then the point was marked on the eggshell using a carbon marker. Fifty eggs were punched at the marked points using an 18-ga needle, and then methylene blue dye (0.05 ml per egg) was injected through the holes using 1-ml insulin syringes equipped with 15-mm (25-ga) disposable needles. Immediately after the injections, windows were made at the blunt end of eggshells, and the air sac membranes were removed using a forceps to identify the delivery sites of dye. Dye traces were detected in amniotic cavity, allantoic space and embryo body in 48%, 26% and 26% of the injected eggs respectively. According to these findings and to eliminate any physical injury to the embryo body, another set of 50 eggs was treated in the same way described above with the exception that the injection was performed at a point approximately 5 mm upper than the former

one, towards the centre of the air sac. The results showed that the dye was loaded into the amniotic cavity, allantoic space and embryo body in 65%, 30% and 5% of the eggs respectively. Finally, 13-mm blunt tip needles (25-ga) were used for dye injection to the remaining 100 eggs through the latter injection point, and loading rates were found to be 68% and 32% in the amniotic cavity and allantoic space respectively with no visible trace under the embryos skins. The latter injection procedure was applied in our further trial. It should be noted that the syringe body was parallel to the long axis of the egg during the injection process.

2.3 | Preparation of injection solutions

Clear moulded glass vials with rubber stoppers and aluminium caps were used to prepare the injection solutions. All vials were autoclaved at 121°C for 20 min before being used. Solutions were prepared in separate clean beakers and were loaded into labelled foil-coated vials through syringes equipped with 0.22-μm syringe filters (MS® CA Syringe Filter, China). The vials were kept at 4°C until injection. To avoid sudden thermal shock, the vials were placed in the incubator to reach the level of incubation temperature (37°C to 38°C), approximately 5 min prior to the injection.

2.4 | Eggs, incubation and injections

Fertile eggs with an average weight of 56.90 ± 1.68 grams (mean ± *SD*) were obtained from a 27-week-old commercial broiler breeder flock and incubated in a commercial hatchery complex (Fariman hatchery Inc., Fariman, Mashhad, Iran). During the first 18 days of incubation period, temperature (T) and relative humidity (RH) were maintained at 37.6°C and 65% respectively. On day 18 of incubation, a total of 480 eggs containing live embryos was candled to determine the injection sites as described previously. Then, the eggs were divided into six groups of four replicates with 20 eggs each and treated as follows: (i) non-injected intact eggs (control); (ii) eggs injected with 0.05 ml distilled water (vehicle control for ascorbic acid); (iii) eggs injected with 0.05 ml distilled water containing 6 mg ascorbic acid (Merck KGaA, Germany, CAS. No. 50-81-7); (iv) eggs injected with 0.05 ml dimethyl sulfoxide (DMSO: vehicle control for chrysin and quercetin; Merck KGaA, Germany, CAS. No. 67-68-5); (v) eggs injected with 0.05 ml DMSO containing 4.5 mg chrysin (97% purity, Sigma-Aldrich Chemie GmbH, Steinheim, Germany, CAS. No. 480-40-0); and (vi) eggs injected with 0.05 ml DMSO containing 4.5 mg quercetin hydrate (≥ 95% purity, Sigma-Aldrich Chemie GmbH, Steinheim, Germany, CAS. No. 849061-97-8.). Shell surface was disinfected by ethanol 70% at the injection site prior to punching. Immediately following the injections, all injected eggs were sealed using a glue gun and subsequently returned to the incubator together with the un-injected control ones. It should be noted that the injection process was implemented in a setter room under standard incubation conditions (T = 37.6 C°; RH=65%). At the end of day 18 of incubation, the eggs were placed in four hatchery trays, each of which had been partitioned into six sections so that each tray contained one replicate of 20 eggs from each treatment. Then, the trays were transferred to a hatching cabinet (T = 37.1°C; RH=75%).

2.5 | Housing conditions and diet

Hatchlings of each in ovo treatment group were mixed together and feather sexed. Then, sixty chicks per treatment were allocated to six replicate floor pens (1 m × 1 m) on fresh wood shavings in a way that each pen accommodated five females and five males. All birds were fed with a standard starter diet for 11 days. The diet (Table 1)

TABLE 1 Ingredients and nutrient composition of the diet fed to chicks from 0 to 11 d of age

Ingredients	Proportions (%)	
Corn	50.01	
Soybean meal (44% CP)	40.92	
Vegetable oil	4.57	
Common salt	0.37	
Calcium carbonate	1.01	
Dicalcium phosphate	1.89	
Vitamin premix[a]	0.25	
Mineral premix[b]	0.25	
DL-methionine (99%)	0.37	
L-lysine HCl (79%)	0.25	
L-threonine	0.11	
Sum	100	
Nutrient composition (%)	**Calculated[c]**	**Analysed[d]**
Metabolisable energy (Kcal/Kg)	2970	–
Crude protein (N × 6.25)	22.77	22.04
Calcium	0.95	–
Available phosphorus	0.48	0.47
Sodium	0.16	–
Methionine	0.71	0.69
Methionine + Cystine	1.07	1.04
Lysine	1.43	1.4
Threonine	0.96	0.95
DCAD (mEq/Kg)[e]	245	–

[a]Provided the followings per kilogram of diet: Vitamin A, 8,800 IU (retinyl acetate); Vitamin D_3, 2,500 IU; Vitamin E, 11 IU (DL-α-tocopheryl acetate); Vitamin K_3, 1.94 mg; B1, 1.49 mg; B2, 4 mg; B3,34.65 mg; B5, 7.92 mg; B6, 2.23 mg; B9, 0.48 mg; B12, 0.01 mg; H_2, 0.15 mg; choline chloride, 140.63 mg; antioxidant, 1 mg.

[b]Provided the followings per kilogram of diet: Mn, 75.15 mg; Fe, 75 mg; Zn, 65.45 mg; Cu, 6.01 mg; I, 0.86 mg; Se, 0.2 mg; choline chloride, 140.63 mg.

[c]Nutrient compositions of feed ingredients were obtained from NRC (1994).

[d]Representative samples were taken from the corn and soybean meal batches used in this study and sent to Evonik Industries AG animal nutrition analytical laboratory (AminoNIR) for the analysis of crude protein, amino acid and available phosphorous contents.

[e]Dietary cation-anion difference.

was formulated to meet Ross 308 nutrient requirements (Aviagen, 2014a). Feed and water were provided ad libitum through a plastic hanging feeder and three nipple drinkers per pen. Management practices were implemented according to the recommendations (Aviagen, 2014b).

2.6 | Evaluated variables

2.6.1 | On-hatch measurements

Hatchlings of each replicate group of eggs were counted and weighed at the end of day 21 of incubation and transferred to the broiler house (poultry research centre of the Ferdowsi University of Mashhad, Mashhad, Iran). Hatchability rate of each replicate group was calculated as follows:

$$\textit{Hatchability rate}\,(\%) = \frac{\text{number of hatchlings in a replicate} \times 100}{\text{number of eggs assigned to the replicate (20 eggs)}}$$

The total hatch weight of each replicate was divided by the number of hatchlings of that replicate to calculate average initial body weight. Six female chicks from each treatment were weighed and killed by cervical dislocation to determine residual yolk sac weight, yolk sac-free body weight and liver weight. Furthermore, the right liver lobes were removed and transferred into 1.5-ml microcentrifuge tubes and stored at −80°C for further analysis. Females were preferred for slaughter for two reasons. First, sex ratios favoured females in DMSO and quercetin groups, both of which also had the lowest hatchability rates (92.50%=72 hatchlings), whereas each treatment was supposed to have six replicates of 10 chicks each with a 1:1 sex ratio to avoid sex effects on performance variables during the rearing phase. Then, all males were retained for the post-hatch growth study, and killed birds were chosen from female hatchlings because their proportions were not limiting in any treatment group. Second, chicks of only one sex (preferably female) were chosen for slaughter to eliminate sex effects on somatic traits because somatic traits of day-old chicks may be influenced by sex (Burke, 1992).

2.6.2 | Assay of hepatic antioxidant indices

The frozen liver samples were homogenised in a potassium chloride (KCl; ≥ 99% purity, Sigma-Aldrich Chemie GmbH, Steinheim, Germany, CAS. No. 7447-40-7) solution (1.15% w/v, pH: 7.4) at 4°C. The homogenates were centrifuged using a refrigerated centrifuge (at 2292 *g* for 15 min). The supernatants were collected and analysed for GPX and total superoxide dismutase (TSOD) activities as well as for total protein and malondialdehyde (MDA) concentrations. The activity of TSOD was assessed colorimetrically (Auto-Analyzer, ALCYON 300-Abbott, USA) using a Ransod kit (Randox Laboratories Ltd. UK) according to Habibi, Sadeghi, and Karimi (2014). An enzymatic method (auto-analyser, ALCYON 300-Abbott, USA) with a Ransel kit (Randox Laboratories Ltd. UK) was used for measuring GPX activity (Paglia & Valentine, 1967). Liver MDA concentration was measured spectrophotometrically at 532 nm (UV-2100, Unico Instruments, Shanghai, China) using the thiobarbituric acid reactive substances (TBARS) method (Satoh, 1978). The spectrophotometric assay described by Bradford (1976) was executed for assessing tissue protein concentration. The TSOD and the GPX activities were reported as unit per mg of protein, and the MDA concentration was expressed as nmol per mg of protein.

2.6.3 | Performance measurements

Pen weights were measured after 3-hr starvation on day 11 and subtracted from the associated initial pen weights to determine chicks weight gains. The subtraction of residual feed from the offered feed in each pen during the 11-day period was used to calculate feed intake. Mortality records were used to correct the feed intake and to calculate the corrected feed to gain ratio (FCR) for each replicate pen.

2.6.4 | Statistical analysis

Data were analysed using the general linear model (GLM) procedure of SAS (SAS Institute (2003)). Analysis of variance showed no differences between blocks (hatchery trays) for all parameters; furthermore, the calculated efficiencies of the randomised complete block design relative to the completely randomised design were less than 100% (Myers & Well, 2003); thus, all data were analysed using a completely randomised design. Tukey's test was used for means separation at $p < .05$. Additionally, a specific orthogonal contrast was performed between the injected groups and non-injected control group for hatchability rate to evaluate the safeness of in ovo injection procedure developed during this study.

3 | RESULTS

3.1 | On-hatch measurements

The effects of in ovo injection of distilled water, ascorbic acid, dimethyl sulfoxide, chrysin and quercetin on hatchability rate and hatch weight are shown in Table 2. Intra-egg administration of chrysin, quercetin or ascorbic did not have an effect on hatchability rate and hatch weight.

The orthogonal contrast for hatchability between the injected and non-injected eggs revealed that in ovo injection led to a significant improvement in hatchability rate (95.25% vs 95%; $p = .0493$) regardless of the type of injected solution, confirming the harmlessness of our injection procedure developed in this study.

The effects of in ovo injection of distilled water, ascorbic acid, dimethyl sulfoxide, chrysin and quercetin on residual yolk sac weight, liver weight and yolk-free body weight of newly hatched broilers are shown in Table 3. Neither injection of chrysin/quercetin nor ascorbic acid into eggs affected residual yolk sac weight, liver weight and yolk-free body weight of newly hatched broilers.

TABLE 2 The effect of in ovo injection of distilled water, ascorbic acid, dimethyl sulfoxide, chrysin and quercetin on hatchability rate and hatch weight of broiler chicks

Injection solutions	Hatchability[a] (%)	Hatch weight[a] (g/bird)
No injection (Control)	95.00	37.375
Distilled water	97.50	36.725
Ascorbic acid	95.00	37.150
Dimethyl sulfoxide	92.50	36.475
Quercetin	92.50	36.675
Chrysin	98.75	36.525
SEM[b]	1.840	0.141
p-value	.1403	.387
Contrast for hatchability		**p-value**
Injected eggs (95.25%) vs un-injected eggs (95%)		.0493[c]

[a]Each mean represents four replicates of 20 eggs.
[b]Standard error of the mean.
[c]Significant at .05 probability level.

TSOD activities and also MDA concentrations were not affected by the in ovo injection of chrysin, quercetin or ascorbic acid.

3.2 | Performance measurements

The effect of in ovo injection of distilled water, ascorbic acid, dimethyl sulfoxide, chrysin and quercetin on the post-hatch performance of broiler chicks from 0 to 11 days of age is shown in Table 5. In ovo injection of chrysin, quercetin or ascorbic acid did not have a significant effect on the early post-hatch performance.

4 | DISCUSSION

4.1 | On-hatch measurements

Hatchability rate and hatch weight were not affected by in ovo injection of chrysin, quercetin or ascorbic acid. Similarly, Nowaczewski, Kontecka, and Krystianiak (2012) reported no significant differences in hatchability between non-injected control eggs and those receiving

TABLE 3 The effect of in ovo injection of distilled water, ascorbic acid, dimethyl sulfoxide, chrysin, and quercetin on yolk, liver and yolk free body relative weights of newly hatched broiler chicks

	Yolk	Yolk free body		Liver
Injection solutions[a]	% of live body weight		Liver	% of yolk free body weight
No injection (Control)	6.61	93.39	3.53	3.78
Distilled Water	5.31	94.69	3.38	3.57
Ascorbic acid	5.23	94.77	3.41	3.59
Dimethyl sulfoxide	5.24	94.76	3.78	3.99
Quercetin	5.72	94.29	3.82	4.04
Chrysin	5.82	94.18	3.54	3.75
SEM[b]	0.684	0.684	0.181	0.178
p-value	.6988	.6988	.4307	.3300

[a]Each mean represents six observations with the exception of liver weight for the dimethyl sulfoxide injected group which was obtained from five observations.
[b]Standard error of the mean.

TABLE 4 The effect of in ovo injection of distilled water, ascorbic acid, dimethyl sulfoxide, chrysin and quercetin on hepatic glutathione peroxidase (GPX) and total superoxide dismutase (TSOD) activities and malondialdehyde (MDA) concentration of newly hatched broiler chicks[a]

Injection solutions	GPX (unit/mg protein)	TSOD (unit/mg protein)	MDA (nmol/mg protein)
No injection (Control)	0.47	2.080	0.210
Distilled water	0.49	2.033	0.215
Ascorbic acid	0.58	2.003	0.217
Dimethyl sulfoxide	0.44	2.297	0.227
Quercetin	0.51	1.983	0.220
Chrysin	0.50	2.012	0.195
SEM[b]	0.041	0.0866	0.0108
p-value	.2615	.1392	.4355

[a]Each mean represents six observations.
[b]Standard error of the mean.

The effects of in ovo injection of distilled water, ascorbic acid, dimethyl sulfoxide, chrysin and quercetin on hepatic GPX and TSOD activities and MDA concentration are shown in Table 4. Liver GPX and

different doses of vitamin C. Hajati et al. (2014) also observed no differences in hatch weight between hatchlings of non-injected eggs and those injected with vitamin C (3 mg/egg) or grape seed extract

TABLE 5 The effect of in ovo injection of distilled water, ascorbic acid, dimethyl sulfoxide, chrysin and quercetin on post-hatch performance of broiler chicks from 0 to 11 day of age[a]

Injection solutions	11-day Body weight (g)	Weight gain (g/bird)	Feed intake (g/bird)	Feed conversion ratio (g: g)
Control	185.20	145.07	218.02	1.508
Distilled Water	172.97	133.52	211.93	1.590
Ascorbic acid	186.34	147.26	219.54	1.495
Dimethyl sulfoxide	186.65	148.12	223.57	1.515
Quercetin	180.43	141.96	219.80	1.555
Chrysin	189.22	150.04	225.61	1.510
SEM[b]	5.939	5.938	6.179	0.0293
p-value	.4425	.4271	.7061	.2112

[a]Each mean represents six observations.
[b]Standard error of the mean.

(4.5 mg/egg); however, the authors recorded higher hatchability rates in eggs injected with vitamin C and grape seed extract as compared to their non-injected counterparts.

The improved overall hatchability rate in injected eggs compared to the non-injected ones in the present study may be, in part, attributed to the possible increase in embryonic metabolic rate due to the mild stress caused by injection. The higher metabolic rate is usually accompanied by an increased respiration rate, resulting in decreased O2 supply and increased CO2 concentrations inside the egg. It has previously been reported that high CO2 conditions during either the early or late period of development accelerate hatching and may lead to an increased hatchability rate (De Smit et al., 2006; Everaert et al., 2007).

The lack of significant changes in yolk sac weight, yolk sac free body weight and liver weight in response to the injected materials in our study is in agreement with observations of Hajati et al. (2014) who found no differences in hatch weight and residual yolk sac weight among chicks hatched from eggs injected with different doses of grape seed extract or vitamin C and those hatched from non-injected eggs.

Our findings regarding liver GPX and TSOD activities and MDA concentration were in contrast to the results of Hajati et al. (2014) and Sirovina et al. (2013). The former reported that in ovo injection of vitamin C at a rate of 3 mg per egg elevated GPX activity. Sirovina et al. (2013) found that both chrysin and quercetin exhibited high antioxidant activities and strong hepatoprotective effects when they were given intraperitoneally for 7 days to diabetic mice. According to these authors, diabetic mice treated with the two flavonoids showed significant reductions in hepatic lipid peroxidation levels evaluated by measuring the malondialdehyde production (Sirovina et al., 2013).

It has been well documented that the antioxidant profile of animal tissues is weakened by ageing; in other words, biomarkers of oxidative stress have been demonstrated to be exacerbated as a function of age (Grune, 2000). This fact has also been confirmed in different avian species. For example, Breque, Surai, and Brillard (2006) found that the antioxidant capacity of uterovaginal junction tissues (sperm storage site in hen's reproductive tract) had a negative correlation with the age of hens. In another study, embryos in eggs produced by an old breeder flock (53 weeks) had a higher heat production rate from day 16 of incubation onward than those in eggs produced by a younger parents stock (29 weeks) (Nangsuay, Meijerhof, Ruangpanit, Kemp, & van den Brand, 2013). Considering the fact that the more heat is produced the more risk of heat stress is expected (Hulet, 2007), it may be concluded that the developing embryos in eggs laid at the late stages of production cycle may be more susceptible to oxidative stress than those develop in eggs laid at an early stage of production. Accordingly, the lack of substantial beneficial effects of the antioxidants evaluated in the present study may be related to the young age (27 weeks) of the parent stock from which the experimental eggs were provided.

4.2 | Performance measurements

Owing to the adverse effect of oxidative stress on muscle growth as well as on the fate of skeletal muscle stem cells (satellite cells; Choi, Ow, Yang, & Taneja, 2016) along with the important role of satellite cells in post-hatch growth of the animal and limited post-hatch proliferative capacity of these cells (Mozdziak, Schultz, & Cassens, 1994), it could be concluded that the alleviation of the hatch-related oxidative stress may lead to a higher hatch weight and post-hatch performance through protection of myofibers and satellite cells from oxidative damages. We did not observe any positive effect of in ovo injection of chrysin, quercetin or ascorbic acid as antioxidant agents on initial and 11-d body weight of broiler chicks. These findings can be justified by our results indicating the lack of significant effect of the three antioxidants on hepatic oxidative status; probably, the severity of hatch-related oxidative stress was not as high as that needed to make the embryo/chick to respond to an exogenous antioxidant supply. Contrary to these results, in ovo injection of ascorbic acid (3 mg/egg) resulted in an improved serum antioxidant status accompanied by an increased FI of broiler chicks from 0 to 10 days of age (Hajati et al., 2014). The authors also demonstrated that the vitamin C injection into eggs tended to enhance weight gain when compared to sham (punctured without injection) and vehicle (punctured and normal saline injected) controls, but the feed conversion ratio remained unaffected.

5 | CONCLUSIONS

It is concluded that in ovo administration of chrysin, quercetin or ascorbic acid at the rates used in the present study did not have a

significant effect on hatchability rate, yolk sac per cent, yolk-free body weight, hepatic enzymatic antioxidant activities, MDA concentration and post-hatch performance of broiler chicks during 0 to 11 d of age. In addition, the harmlessness of the injection procedure developed during this study was approved.

Owing to the negative relationship between animal age and body antioxidant status, further studies are needed to evaluate the interactions between breeder age and in ovo injection of exogenous antioxidants on different characteristics of broiler chicks.

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REFERENCES

Anand, K. V., Mohamed Jaabir, M. S., Thomas, P. A., & Geraldine, P. (2012). Protective role of chrysin against oxidative stress in d-galactose-induced aging in an experimental rat model. *Geriatrics and Gerontology International*, *12*, 741–750.

Aviagen. (2014a). *Ross 308: Broiler nutrition specifications*. Huntsville, Alabama, USA: Aviagen. http://www.aviagen.com

Aviagen. (2014b). *Ross 308: Broiler management handbook*. Huntsville, Alabama, USA: Aviagen. http://www.aviagen.com

Bender, D. A. (2003). *Nutritional biochemistry of the vitamins*, 2nd ed.. NY, USA: Cambridge University Press.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*, 248–254.

Breque, C., Surai, P., & Brillard, J. P. (2006). Antioxidant status of the lower oviduct in the chicken varies with age and dietary vitamin E supplementation. *Molecular Reproduction and Development*, *73*, 1045–1051.

Burke, W. H. (1992). Sex differences in incubation length and hatching weights of broiler chicks. *Poultry Science*, *71*, 1933–1938.

Choi, M. H., Ow, J. R., Yang, N.-D., & Taneja, R. (2016). *Oxidative stress-mediated skeletal muscle degeneration: molecules, mechanisms, and therapies. Oxidative medicine and cellular longevity*. 2016, Article ID 6842568, 13 pages. https://doi.org/10.1155/2016/6842568

De Smit, L., Bruggeman, V., Tona, J. K., Debonne, M., Onagbesan, O., Arckens, L., … Decuypere, E. (2006). Embryonic developmental plasticity of the chick: Increased CO2 during early stages of incubation changes the developmental trajectories during prenatal and postnatal growth. *Comparative Biochemistry and Physiology*, *145A*, 166–175.

Ebadi, M. (2007). *Pharmacodynamic basis of herbal medicine*, 2nd ed.. Boca Raton, London, New York: CRC Press, Taylor and Francis Group.

Everaert, N., Kamers, B., Witters, A., De Smit, L., Debonne, M., Decuypere, E., & Bruggeman, V. (2007). Effect of four percent carbon dioxide during the second half of incubation on embryonic development, hatching parameters, and posthatch growth. *Poultry Science*, *86*, 1372–1379.

Favero, A., Vieira, S. L., Angel, C. R., Bos-Mikich, A., Lothhammer, N., Taschetto, D., … Ward, T. L. (2013). Development of bone in chick embryos from Cobb 500 breeder hens fed diets supplemented with zinc, manganese, and copper from inorganic and amino acid-complexed sources. *Poultry Science*, *92*, 402–411.

Grune, T. (2000). Oxidative stress, aging and the proteasomal system. *Biogerontology*, *1*, 31–40.

Habibi, R., Sadeghi, G., & Karimi, A. (2014). Effect of different concentrations of ginger root powder and its essential oil on growth performance, serum metabolites and antioxidant status in broiler chicks under heat stress. *British Poultry Science*, *55*, 228–237.

Hajati, H., Hassanabadi, A., Golian, A., NassiriMoghaddam, H., & Nassiri, M. R. (2014). The effect of in ovo injection of grape seed extract and vitamin C on hatchability, antioxidant activity, yolk sac weight, performance and ileal micro flora of broiler chickens. *Research Opinions in Animal and Veterinary Sciences*, *4*, 633–638.

Hulet, R. M. (2007). Symposium: Managing the embryo for performance managing incubation: Where are we and why? *Poultry Science*, *86*, 1017–1019.

Kadam, M. M., Barekatain, M. R., K-Bhanja, S., & Iji, P. A. (2013). Prospects of in ovo feeding and nutrient supplementation for poultry: The science and commercial applications-a review. *Journal of the Science of Food and Agriculture*, *93*, 3654–3661.

Lee, S. H., Lillehoj, H. S., Jang, S. I., Jeong, M. S., Xu, S. Z., Kim, J. B., … Bravo, D. M. (2014). Effects of in ovo injection with selenium on immune and antioxidant responses during experimental necrotic enteritis in broiler chickens. *Poultry Science*, *93*, 1113–1121.

Liu, H. N., Liu, Y., Hu, L. L., Sou, Y. L., Zhang, L., Jin, F., … Li, Y. (2014). Effects of dietary supplementation of quercetin on performance, egg quality, cecal microflora populations, and antioxidant status in laying hens. *Poultry Science*, *93*, 347–353.

Malheiros, R. D., Ferket, P. R., & Gonçalves, F. M. (2012). *Oxidative stress protection of embryos by "in ovo" supplementation*. Salvador - Bahia – Brazil: World's Poultry Congress.

Moghaddam, A., Karimi, I., Borji, M., Bahadori, S., & Abdolmohammadi, A. (2013). Effect of royal jelly in ovo injection on embryonic growth, hatchability, and gonadotropin levels of pullet breeder chicks. *Theriogenology*, *80*, 193–198.

Mozdziak, P. E., Schultz, E., & Cassens, R. G. (1994). Satellite cell mitotic activity in post-hatch turkey skeletal muscle growth. *Poultry Science*, *73*, 547–555.

Murphy, K. J., Chronopoulos, A. K., Singh, I., Francis, M. A., Moriarty, H., Pike, M. J., … Sinclair, A. J. (2003). Dietary flavanols and procyanidin oligomers from cocoa (Theobroma cacao) inhibit platelet function. *The American Journal of Clinical Nutrition*, *77*, 1466–1473.

Myers, J. L., & Well, A. D. (2003). *Research design and statistical analysis*, 2nd ed.. Mahwah, New Jersey, London: Lawrence Erlbaum Associates Publishers.

Nangsuay, A., Meijerhof, R., Ruangpanit, Y., Kemp, B., & van den Brand, H. (2013). Energy utilization and heat production of embryos from eggs originating from young and old broiler breeder flocks. *Poultry Science*, *92*, 474–482.

Nijveldt, R. J., van Nood, E., van Hoorn, D. E. C., Boelens, P. G., van Norren, K., & van Leeuwen, P. A. M. (2001). Flavonoids: A review of probable mechanisms of action and potential applications. *American Journal Clinical Nutrition*, *74*, 418–425.

Nowaczewski, S., Kontecka, H., & Krystianiak, S. (2012). Effect of in ovo injection of vitamin C during incubation on hatchability of chickens and ducks. *Folia Biologica (Kraków)*, *60*, 93–97.

Paglia, D. E., & Valentine, W. N. (1967). Studies on quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *Journal of Laboratory and Clinical Medicine*, *70*, 158–169.

Rapta, P., Misík, V., Stasko, A., & Vrábel, I. (1995). Redox intermediates of flavonoids and caffeic acid esters from propolis: An EPR spectroscopy and cyclic voltammetry study. *Free Radical Biology and Medicine*, *18*, 901–908.

Rinaldini, L. M. (1960). Growth and ascorbic acid content of the chick embryo. *Journal of Embryology & Experimental Morphology*, *8*, 527–539.

SAS Institute. (2003). *SAS system for microsoft windows. Version 9.1*. Cary, NC, USA: SAS Institute.

Satoh, K. (1978). Serum lipid peroxide in cerebrovascular disorders determined by a new colorimetric method. *Clinica Chimica Acta*, *90*, 37–43.

Sirovina, D., Orsolić, N., Koncić, M. Z., Kovacević, G., Benković, V., & Gregorović, G. (2013). Quercetin vs chrysin: Effect on liver histopathology in diabetic mice. *Human and Experimental Toxicology*, *32*, 1058–1066.

Soleimani, A. F., Zulkifli, I., Hair-Bejo, M., Omar, A. R., & Raha, A. R. (2012). The role of heat shock protein 70 in resistance to *Salmonella enteritidis* in broiler chickens subjected to neonatal feed restriction and thermal stress. *Poultry Science*, *91*, 340–345.

Surai, P. F., & Fisinin, V. L. (2012). *Feeding breeders to avoid oxidative stress in embryos*. Salvador - Bahia – Brazil: World's Poultry Congress.

Surai, P. F., Noble, R. C., & Speake, B. K. (1996). Tissue-specific differences in antioxidant distribution and susceptibility to lipid peroxidation during development of the chick embryo. *Biochimica et Biophysica Acta*, *1304*, 1–10.

Uni, Z., Yadgary, L., & Yair, R. (2012). Nutritional limitations during poultry embryonic development. *Journal of Applied Poultry Research*, *21*, 175–184.

Urso, U. R. A., Dahlke, F., Maiorka, A., Bueno, I. J. M., Schneider, A. F., Surek, D., & Rocha, C. (2015). Vitamin E and selenium in broiler breeder diets: Effect on live performance, hatching process, and chick quality. *Poultry Science*, *94*, 976–983.

Vassallo, N. (2008). *Polyphenols and health: New and recent advances*. New York: Nova Science Publishers.

Williams, C. A., Harborne, J. B., Newman, M., Greenham, J., & Eagles, J. (1997). Chrysin and other leaf exudate flavonoids in the genus Pelargonium. *Phytochemistry*, *46*, 1349–1353.

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