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Effects of In-Ovo Rutin Injection to Fertile Japanese Quail (Coturnix Coturnix Japonica) Egg on Hatchability, Embryonic Death, Hatchling Weight, and Hatchling Liver Oxidative and Nitrosative Stress

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ABSTRACT

This study was conducted with the aim of investigating the effects of the antioxidant rutin injected in fertilized quail eggs on incubation parameters and some hatchling liver biochemical parameters. The study was carried out with 6 groups including a control group and 5 different doses of rutin, and it involved 720 fresh Japanese quail (*Coturnix coturnix japonica*) eggs. It was observed that rutin dose did not affect the early embryo mortality, whereas intermediate and late embryo mortality rates were higher in all groups given rutin in comparison to the control group. The mean hatchability of fertile eggs and total eggs for the control, 0.25 mg, 0.50 mg, 0.75 mg, 1 mg and 1.5 mg groups were calculated as 82.06, 82.23, 64.43, 68.84, 44.08, 22.95 % and 48.10, 55.49, 34.33, 33.00, 18.03, 8.45% respectively. Compared with the control group, hatchling mortality rate was higher only in the 0.25 rutin group, and lower in all other groups receiving rutin *in-ovo*. The highest hatchling weight was found in the 0.25 mg rutin group, and hatchling weight decreased as rutin dose increased. Consequently, considering the mortality rates, hatchling weights, and liver antioxidant/oxidant capacities of the hatchlings, it is believed that the *in-ovo* injection of 0.25 mg rutin may be useful for Japanese quail production.

INTRODUCTION

The poultry industry is becoming highly and increasingly significant to overcome the protein deficit in developing countries. Reaching the desired success in this sector is dependent on daily chick production [28]. Due to the high metabolic rate of embryos as a result of intensive selection applied on commercial poultry, their nutritional requirements have also increased. Failing to meet these requirements nutrition has negative effects on parameters such as embryonic development, hatchability, hatchling quality, and post-hatch performance [24]. This situation has led to an increase in the number of studies aiming at increasing incubation yield and reducing post-hatch disease and mortality rates. In this context, in-egg (*in-ovo*) feeding has attracted attention of researchers [9], and most of the studies have evaluated the injection of nutrients, such as glucose, amino acids, trace minerals, essential fatty acids and vitamins into the amnion and yolk sac of poultry embryos [5, 6, 16, 26, 32, 33, 35, 43, 46].

In addition to well-known antioxidant vitamins (vitamins A and C), foods contain some compounds have equally effective antioxidant properties, such as flavonoids. These compounds have anticancer and other useful properties are also known as dietary antioxidants. Over 4000 flavonoids have been evaluated as dietary antioxidants, including rutin [21]. Rutin, which is found in fruits, vegetables and herbal teas, is a non-toxic substance that consists of the flavonol quercetin and the disaccharide rutinose, and has antioxidant, anti-inflammatory, and

antidiabetic activities [3, 14, 37, 44]. In the light of this information, this study was carried out with the purpose of investigating the effects of the *in-ovo* injection of fertilized quail eggs with rutin on embryonic death, chick death, hatchability, hatchling weight, and some biochemical parameters.

MATERIALS AND METHODS

Location

The experiment was conducted at the Poultry Unit of the Department of Husbandry Research and Application of the School of Veterinary Medicine, Atatürk University, Erzurum, Turkey. The study was conducted in accordance with ethical rules and procedures, and was approved by Atatürk University

Local Ethics Council of Animal Experiments (19.04.2016/2).

Chemicals

The rutin and other chemicals used in this study were of analytical purity and were purchased from Sigma-Aldrich (St Louis, MO, USA).

Experimental procedures

A total of 778 eggs were obtained from 16- to 20-wk-old 240 (120 male and 120 female) Japanese quail breeders (*Coturnix coturnix japonica*) in a total of 7 days. The eggs were stored in a room at 18-20°C and 55-60% relative humidity until incubation. Quails housed in 120 multi-story breeding cages (one male and one female per cage), and fed with a diet containing 20% crude protein and 2900 kcal/kg metabolizable energy. Birds were randomly distributed into six groups, with three replicates of 40 eggs each. The following *in-ovo* injection treatments were applied: T1 (Control): 0.1 mL of physiological saline solution; T2: 0.25 mg rutin/10 g egg; t3: 0.50 mg rutin/10 g egg; t4: 0.75 mg rutin/10 g egg; t5: 1.00 mg rutin/10 g egg; and T6: 1.50 mg rutin/10 g egg.

Rutin doses was adjusted per 10 g of egg weight and dissolved in physiological saline solution to a final volume of 1 mL. It is understood from the literature reviews that *in-ovo* injection into quail eggs is made into the air sac. Eggs were disinfected with ethanol at 70% and pierced on the flatter end (air cell) of the eggs. The solutions were manually injected using a 26 G syringe at an approximate 5-mm depth before incubation. After injection, the holes were covered by nail polish, and again disinfected with ethanol at 70%. After completion of the injection process, a total of 720 Japanese quail eggs were set in a single-stage incubator. The relative humidity and temperature in the setter (0–14 days) were 68% and 37.8 °C, respectively, and in the hatcher (15–17 days), 78% and 36.8 °C, respectively. At hatch, after the down was dried, hatchlings were individually weighed to calculate average body weight (BW). Ten hatchlings per treatment were decapitated after mild sevoflurane anesthesia. Hatchling liver samples were collected and frozen at -20ºC until biochemical analyses.

Unhatched eggs were broken, and the number of infertile eggs was counted. Embryo mortality was classified according to incubation stages as early (1 to 6 d), intermediate (7 to 14 d) or late mortality (15 to 18 d). In order to determine incubation results, hatchability of fertile eggs (number of hatchlings/number of fertile eggs), hatchability of total eggs (number of chicks/total of number eggs) and embryo death percentages (%) (early, middle, final) were calculated.

The healthy chicks were reared in separate brooders according to treatment for two weeks, and were checked daily. The number of birds that died during the two-week period were recorded daily and total mortality rate was calculated.

Liver biochemical analyses

Liver tissues were homogenized in a tissue lyser II (Qiagen) homogenizer with a buffer containing 1.15% potassiumchloride (KCl) to obtain 1:10 ratio (w/v) of whole homogenate. Total Antioxidant Capacity (TAC) was determined using TAC assay kit (Rel Assay Diagnostic, Turkey) and expressed in mmol trolox equiv./g tissue. Total Oxidant Capacity (TOC) was calculated by TOC assay kit (Rel Assay Diagnostic, Turkey) and expressed in mmol H_2O_2 equiv./g tissue. Nitric Oxide (NO) levels were determined out by the method of colorimetric determination of nitrite and expressed in nmol/g tissue, which is a colorful azo-dye product of Griess reaction that absorbs visible light at 540 nm after nitrate is enzymatically converted into nitrite by the enzyme nitrate reductase (NO detection kit, Enzo Life Science). Malondialdehyde (MDA) levels in the liver samples were measured spectrophotometrically based on the method modified by Placer *et al.* [34] and expressed in nmol/g tissue. The GSH (glutathione) levels were determined at 412 nm by the method of Sedlak and Lindsay [36] and expressed in nmol/g tissue.

Statistical analysis

Hatchling body weight results were analyzed by one-way analysis of variance. The effects of rutin on

early, intermediate, and late embryo mortality and 2-wk-old chick mortality rates were analyzed using the following logistic regression model:

$P(y)=(1+e^{(\beta_0+\beta_1 X_1)})^{-1}$

Where: (P(z) is the probability of hatched (1) or embryonic death (0);, β_0 = fixed regression coefficient; β_z= Fixed regression coefficient); X_1= rutin doses (control, 0.25, 0.50, 0.75, 1, 1.5 mg)

Liver biochemical results were analyzed according to the Repeated Measures Analysis model:

Yij = μ + Ai +eij

Where: Yij: TAC, TOC, NO, MDA, GSH; μ: overall population mean; Ai: fixed effect of control, 0.25, 0.50, 0.75, 1, 1.5 mg rutin doses; eij: random error term assumed to be normally and independently distributed with mean of zero and variance σ2 e. The SPSS package software was used for all statistical analyses [39].

RESULTS

Incubation parameters

Mean hatchability of fertile eggs and total eggs of the control, 0.25 mg, 0.50 mg, 0.75 mg, 1 mg and 1.5 mg treatments were determined as 82.06, 82.23, 64.43, 68.84, 44.08, 22.95% and 48.10, 55.49, 34.33, 33.00, 18.03, 8.45% respectively, and hatchling weights of 7.89±0.065, 8.20±0.097, 7.83±0.084, 7.87±0.139, 7.68±0.141 and 7.75±0.242 g, respectively (Table 1). The heaviest hatchlings derived from eggs injected with 0.25 mg rutin, and the lightest with 1 and 1.5 mg rutin, while the control, 0.50 and 0.75 mg treatments presented statistically intermediate values. Additionally, it was observed that the hatching weight decreased as rutin dose increased.

Table 1 – Hatchling weight as a function of treatments.

Treatments	n	Hatchling weight	*p* value
Control	58	7.89±0.065^{ab}	0.043
0.25 mg	67	8.20±0.097^{a}	
0.50 mg	41	7.83±0.084^{ab}	
0.75 mg	40	7.87±0.139^{ab}	
1.00 mg	22	7.68±0.141^{b}	
1.50 mg	10	7.75±0.242^{b}	

Eggs injected with 1.00 mg rutin showed the highest early embryo mortality rate, while the highest intermediate and late embryo mortality rates were obtained with the 1.50 mg rutin *in-ovo* injection. Additionally, early and late embryo mortality rates in the 0.25 mg rutin treatments were lower than that of the control group (Table 2).

Table 2 – Early, intermediate, and late embryo mortality rates (%) as a function of treatment.

	Treatments					
Embryo mortality rate (%)	Control	0.25 mg	0.50 mg	0.75 mg	1.00 mg	1.50 mg
Early (1 to 6 d)	5.12	2.70	4.96	2.77	11.03	5.42
Intermediate (7 to 14 d)	1.84	4.83	10.91	9.22	9.14	24.90
Late (15 to 18 d)	10.98	10.24	19.70	19.17	35.75	46.73

The logistic equation results show that rutin injection dose did not influence early embryo mortality rate, but it significantly affected intermediate and late embryo mortality rates (p<0.001) (Table 3). According to Table 3, the early embryo mortality in the eggs that were injected with 0.25 mg, 0.50 mg, 0.75 mg and 1.50 mg rutin were lower with comparison to the control group. Intermediate and late embryo mortality rates in all rutin-injected groups were higher in comparison with the control group, while in the 1.50 rutin group, intermediate embryo mortality rate increased by approximately 9.5 times and late embryo mortality rates increased by approximately 7 times.

Table 4 shows hatchling mortality rate results. Accordingly, only the mortality rates of hatchlings from eggs injected with 0.25 mg rutin were higher than the control group, while the rates in the other groups were all lower in comparison with the control group (p<0.05).

Liver biochemical analysis results

Figures 1-5 show the TAC, TOC, NO, MDA and GSH values determined in the hatchlings' liver. Accordingly, TAC value was higher in all rutin-injected groups in comparison with the control group and the highest mean value was obtained in the 0.25 mg treatment (quadratic p<0.0001 and cubic p<0.0001), whereas the TOC value in the control group was higher those obtained in the rutin-inject groups (quadratic p<0.0001 and cubic p<0.0001). The lowest NO value was obtained in the 0.25 mg rutin group and the highest values in the control group (quadratic p<0.0001 and cubic p<0.01). The lowest MDA value was detected in the 0.25 mg group and the highest in the control group (quadratic p<0.0001 and cubic p<0.0001), the lowest GSH value was analyzed in the dosage of 1 mg, while the values in the control group were lower than the other experiment groups (quadratic p<0.01 and cubic p<0.0001).

Table 3 – Logistic Regression results of embryo mortality rates according to treatment

	Treatment	B	SE	Wald	*p* value	Exp. (B)
Early	Control	1.000	1.000	4.069	0.540	1.000
	0.25 mg	-0.872	0.778	1.258	0.262	0.418
	0.50 mg	-0.576	0.513	1.262	0.261	0.562
	0.75 mg	-1.241	1.054	1.387	0.239	0.289
	1 mg	0.196	0.521	0.142	0.707	1.216
	1.5 mg	-0.164	0.596	0.076	0.783	0.849
	Treatment	B	S.E	Wald	*p* value	Exp. (B)
Intermediate	Control	1.000	1.000	27.798	0.001	1.000
	0.25 mg	0.118	0.706	0.028	0.867	1.125
	0.50 mg	1.178	0.460	6.550	0.010	3.248
	0.75 mg	1.026	0.610	2.831	0.092	2.791
	1 mg	1.083	0.538	4.057	0.044	2.954
	1.5 mg	2.231	0.471	22.429	0.001	9.306
	Treatment	B	S.E	Wald	*p* value	Exp. (B)
Late	Control	1.000	1.000	44.456	0.001	1.000
	0.25 mg	0.017	0.471	0.001	0.970	1.018
	0.50 mg	0.892	0.317	7.896	0.005	2.439
	0.75 mg	0.700	0.446	2.463	0.117	2.013
	1 mg	1.633	0.347	22.095	0.001	5.120
	1.5 mg	1.929	0.349	30.623	0.001	6.886

B: estimated logit coefficient, SE standard error of the coefficient, Wald: $[B/S.E.]^2$, Exp. (B): odds ratio of the individual coefficient.

Table 4 – Logistic Regression results of 2-wk-old chick mortality rates according to treatment.

	Treatment	B	SE	Wald	*p* value	Exp. (B)
Hatchling mortality rate	Control	1.000	1.000	76.888	0.001	1.000
	0.25 mg rutin	0.206	0.368	0.313	0.576	1.229
	0.50 mg rutin	-0.816	0.250	10.664	0.001	0.442
	0.75 mg rutin	-0.528	0.362	2.123	0.145	0.590
	1.00 mg rutin	-1.564	0.300	27.211	0.001	0.209
	1.50 mg rutin	-2.579	0.346	55.498	0.001	0.076

B: estimated logit coefficient, SE: standard error of the coefficient, Wald: $[B/S.E.]^2$, Exp. (B): odds ratio of the individual coefficient.

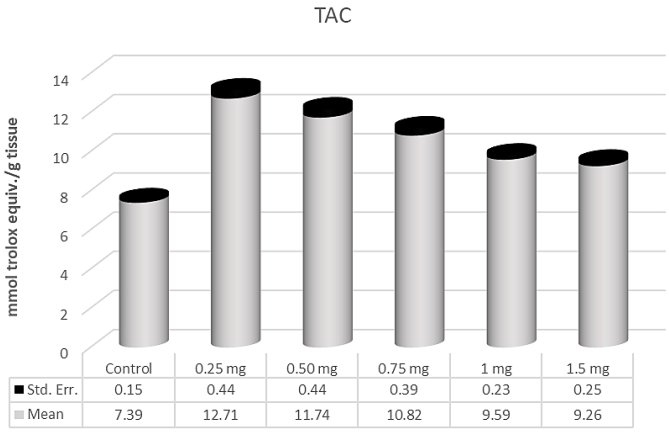

Figure 1 – Total Antioxidant Capacity (TAC) levels in the liver tissue

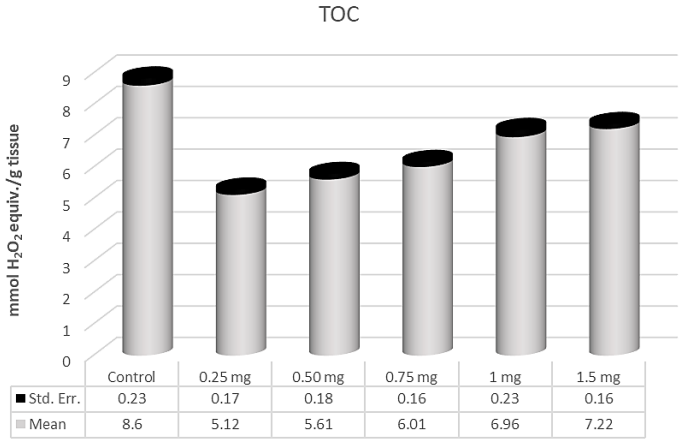

Figure 2 – Total Oxidant Capacity (TOC) levels in the liver tissue

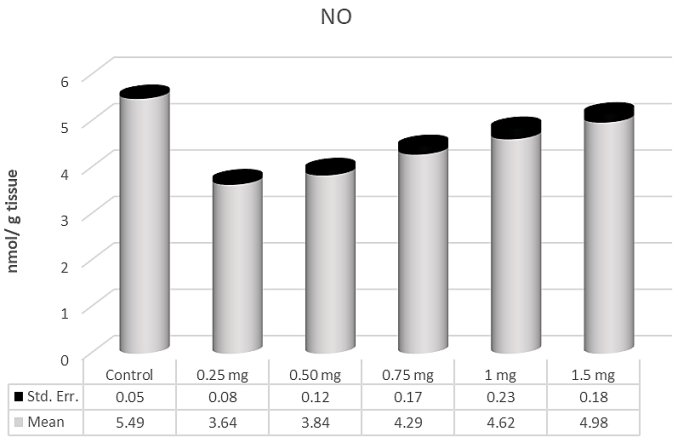

Figure 3 – Nitric Oxide (NO) levels in the liver tissue

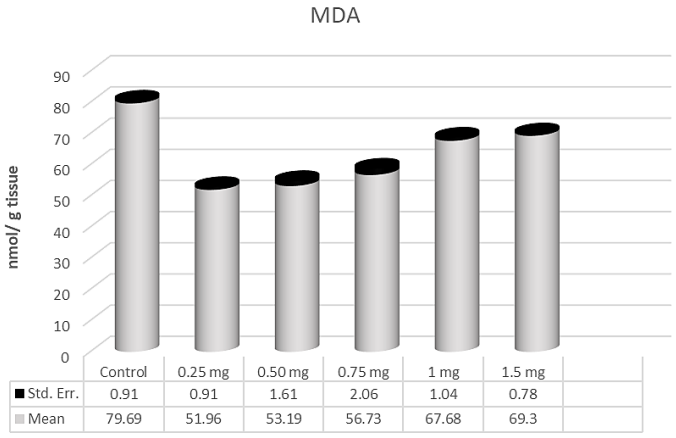

Figure 4 – Malondialdehyde (MDA) levels in the liver tissue

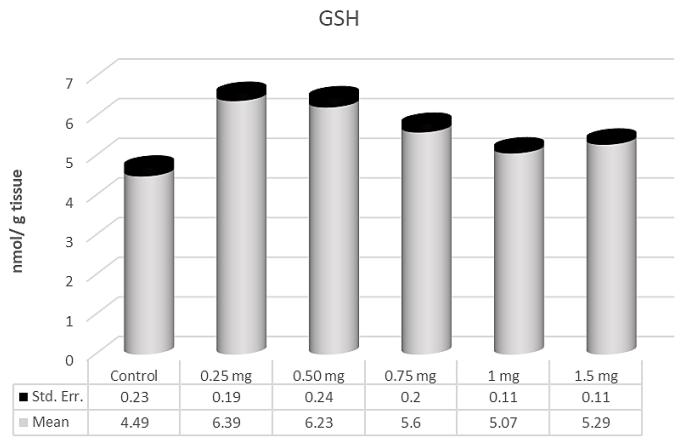

Figure 5 – Glutathione (GSH) levels in the liver tissue

The mean least square results for TAC, TOC, NO, MDA and GSH levels are given in Table 5. Accordingly, all the measured parameters (p<0.001) presented a linear response to *in-ovo* rutin injection, but no significant cubic responses were detected (p>0.05).

DISCUSSION

As the success of the commercial poultry production depends of the breeding sector, incubation results are highly important [15]. Although modern incubation methods are applied, incubation yield losses are still experienced to a certain extent [30]. Various studies have evaluated factors affecting both breeding flocks and eggs for incubation, such as *in-ovo* nutrition. Research has shown that the injection of carbohydrates (glucose, fructose, maltose, sucrose), vitamins (B_1, C, E) and hormone (insulin growth factor) in incubated eggs negatively affected hatchability [8, 16, 29, 35, 46].

In the present study, although the hatchability of the fertilized eggs injected with 0.25 mg rutin was similar to that of the control group, it was reduced 20.6% at higher rutin doses. This result may be explained by the high intermediate and late embryo mortality in eggs injected with rutin. Differently from our findings, *in-ovo* injection of L-carnitine by Keralapurath *et al.* [26], of NaCl by Tangara *et al.* [40], and vitamin C by Ipek *et al.* [20] had positive effects on incubation results, whereas no influence was detected with the *in-ovo* injection of L-carnitine by Zhai *et al.* [47], vitamin D_3 by Bello *et al.* [6], and vitamin C by Nowaczewski *et al.* [32].

The heaviest hatchlings were obtained from eggs injected with 0.25 mg rutin, which, however, were not

Table 5 – Mean least squares results of TAC, TOC, NO, MDA and GSH according to treatment.

PARAMETER	Factor	Type III Sum of Squares	Mean Square	F	Sig.
TAC	Linear	81.939	81.939	112.922	0.001
	Cubic	0.712	0.712	0,316	0.588
TOC	Linear	30.969	30.969	141.257	0.001
	Cubic	0.354	0.354	0.944	0.357
NO	Linear	11.909	11.909	92.800	0.001
	Cubic	0.047	0.047	0.086	0.776
MDA	Linear	2418.181	2418.181	261.598	0.001
	Cubic	135.839	135.839	5.346	0.046
GSH	Linear	11.330	11.330	31.332	0.001
	Cubic	1.457	1.457	4.618	0.060

TAC: Total antioxidant capacity, TOC: Total oxidant capacity, NO: Nitric oxide, MDA: Malondialdehyde, GSH: Glutathione

statistically different from those of the control group. Moreover, increasing rutin doses reduced hatchability ($p<0.05$). Studies on *in-ovo* injection of glucose [35] and NaCl [40] obtained higher hatchling weight than the controls.

A study with mice reported positive effects of antioxidant administration in the early periods of embryonal development on fetal development [41]. In humans, it was found that antioxidants did not have any negative effects on underdeveloped lungs, but they harmed these organs after the lungs started to develop [7]. In poultry, lungs develop in the intermediate and final stages of the incubation period [4]. In the present study, rutin injection dose did not have any significant effect on early embryo mortality rates, but resulted in higher intermediate and late embryo mortality rates at all rutin doses compared with the control group ($p<0.001$), which may have been a result of lung damage caused by antioxidant *in-ovo* injection. In addition, it is hypothesized that some air remained in the air cell after the *in-ovo* injection of rutin, particularly at higher doses, which may have negatively affected embryo mortality rates.

Only the 0.25 rutin group presented higher 2-wk-old chick mortality rate than the control group, while the rates of the other treatments were lower than that in the control group ($p<0.05$).

The biochemical parameter TAC is an indicator of the overall antioxidant status of the serum and body fluids resulting from antioxidant intake and/or production, and their utilization under normal or increased levels of ROS production. It assesses the capacity of known and unknown antioxidants and their synergistic interaction, giving an insight into the delicate balance between oxidants and antioxidants *in vivo* [18]. The antioxidant capacity of tissues can be attributed to individual components of the defense system against free radicals, which can be measured and are used to calculate TAC [23]. The measurement of the total oxidant status (TOC) accurately reflects the oxidative status of the blood plasma or serum. Nitric oxide (NO) acts as a free radical and contributes to host defenses against oxidation. Malondialdehyde (MDA) is a reliable and commonly used marker of overall lipid peroxidation levels and the presence of oxidative stress [25]. Glutathione (GSH), a water-soluble tripeptide composed of the amino acids glutamine and glycine [40], and protects tissues and organs against the adverse effects of reactive oxygen species. GSH plays a role in the elimination of free radical species such as H_2O_2, superoxide radicals and membrane protein tails [10, 11]. The effects of rutin on changes in oxidative stress levels were investigated in different species of animals exposed to viral diseases, bacterial diseases, parasitic infections, skin conditions, chemotherapeutic drugs, antibiotics, various chemical compounds, heavy metals, and temperature stresses. It was shown that rutin reduces oxidative stress and has detoxication, which render it useful for supportive treatment [1, 2, 12, 13, 17, 19, 22, 25, 31, 38, 45]. This study determined that the *in-ovo* injection of rutin had a positive effect on the measure liver antioxidant parameters, reducing the harmful effects of the oxidative stress created by incubation.

CONCLUSION

During the incubation period of poultry, the only source of food for the embryo are the nutrients found in the egg. Therefore, it is believed that changing the nutrient composition of the egg using *in-ovo* applications may be have positive effects both on the embryo and on the resulting chicks. Considering that, compared with *in-ovo* injection of saline solution, the *in-ovo* injection of 0.25 mg rutin promoted lower early embryo mortality rate and similar intermediate and late mortality rates, higher hatchling weight and better antioxidant status. Therefore, the *in-ovo* injection of 0.25 mg rutin will be useful for Japanese quail production.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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