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

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
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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 -20°C 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 H2O2 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 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 antioxidant and nitrosative stress.
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 – Hatching weight as a function of treatments.

| Treatments | n  | Hatching weight | p value  |
|------------|----|----------------|----------|
| Control    | 58 | 7.89±0.065a    |          |
| 0.25 mg    | 67 | 8.20±0.097a    |          |
| 0.50 mg    | 41 | 7.83±0.084a    | 0.043    |
| 0.75 mg    | 40 | 7.87±0.139ab   |          |
| 1.00 mg    | 22 | 7.68±0.141b    |          |
| 1.25 mg    | 10 | 7.75±0.242b    |          |

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.

| 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   |

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<0.0001). 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).

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

\[ Y_{ij} = \mu + A_i + e_{ij} \]

Where: Yi: 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 \( \sigma^2 \). The SPSS package software was used for all statistical analyses [39].
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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 rutin | -0.872 | 0.778 | 1.258 | 0.262 | 0.418    |
| 0.50 mg rutin | -0.576 | 0.513 | 1.262 | 0.261 | 0.562    |
| 0.75 mg rutin | -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    |
| Intermediate |       |     |      |         |          |
| Control     | 1.000 | 1.000 | 27.798 | 0.001 | 1.000    |
| 0.25 mg rutin | 0.118 | 0.706 | 0.028 | 0.867 | 1.125    |
| 0.50 mg rutin | 1.178 | 0.460 | 6.550 | 0.010 | 3.248    |
| 0.75 mg rutin | 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    |
| Late         |       |     |      |         |          |
| Control     | 1.000 | 1.000 | 44.456 | 0.001 | 1.000    |
| 0.25 mg rutin | 0.017 | 0.471 | 0.001 | 0.970 | 1.018    |
| 0.50 mg rutin | 0.892 | 0.317 | 7.896 | 0.005 | 2.439    |
| 0.75 mg rutin | 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 | 4.422    |
| 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 | 5.209    |
| 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.

Table 3 – Logistic Regression results of 2-wk-old chick 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 rutin | -0.872 | 0.778 | 1.258 | 0.262 | 0.418    |
| 0.50 mg rutin | -0.576 | 0.513 | 1.262 | 0.261 | 0.562    |
| 0.75 mg rutin | -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    |
| Intermediate |       |     |      |         |          |
| Control     | 1.000 | 1.000 | 27.798 | 0.001 | 1.000    |
| 0.25 mg rutin | 0.118 | 0.706 | 0.028 | 0.867 | 1.125    |
| 0.50 mg rutin | 1.178 | 0.460 | 6.550 | 0.010 | 3.248    |
| 0.75 mg rutin | 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    |
| Late         |       |     |      |         |          |
| Control     | 1.000 | 1.000 | 44.456 | 0.001 | 1.000    |
| 0.25 mg rutin | 0.017 | 0.471 | 0.001 | 0.970 | 1.018    |
| 0.50 mg rutin | 0.892 | 0.317 | 7.896 | 0.005 | 2.439    |
| 0.75 mg rutin | 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.
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 on 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, 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
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The authors declare that there is no conflict of interest regarding the publication of this paper.

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