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Original article

Determination of the effects of in-ovo chrysin addition to fertile quail eggs on testicular histology, oxidative stress and semen quality

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Abstract

In this study, the effects of *in-ovo* injection of Chrysin (CR) into Japanese quail eggs on testicular histology, oxidant status and epididymal sperm quality were investigated. 720 Japanese quail eggs were divided into 4 groups and 0.1 mL saline was given to the control group, 0.25 mg CR to the 0.25 group, 0.50 mg CR to the 0.50 group and 0.75 mg CR to the 0.75 group. On the 60th day after the laying of eggs, 8 Japanese quails were randomly selected from each group and sacrificed under mild sevoflurane anesthesia. The abdominal cavity was opened and both testicular tissues and epididymal parts were removed. The right testicular tissue was used for histopathological examinations and the left one was used for biochemical analyses. The epididymal part at the tip of the vas deferens at the end of the right testis was trimmed in 100 µL saline at 37°C. Histopathological examinations showed that histological scoring was higher in the CR 50 and CR 75 groups. A decrease in oxidant status was observed in all CR groups compared to the control group. Spermatozoa density was higher in CR groups compared to the control group. Total motility value was statistically significantly higher in CR 50 and CR 75 groups compared to control and CR 25 groups. There was no statistical difference between the groups in terms of dead and abnormal spermatozoa. *In-ovo* CR injection at doses of 0.50 and 0.75 mg/egg improved testicular histological score, decreased oxidative stress, and increased epididymal sperm quality.

Keywords: chrysin, Japanese quail, oxidative stress, sperm, testis

Introduction

Bird species have a limited food supply, which is stored in the egg (Uni et al. 2012). Although nutrient levels in the egg are adequate for embryonic needs, nutritional supplements may be required for optimum growth and development (Kermanshahi et al. 2017a). Therefore, the use of *in-ovo* injection is shown as a way of providing additional nutrients (Kadam et al. 2008, Chen et al. 2010). In-ovo injection is a method used to administer exogenous substances into the egg due to its effects on hatchability, and post-hatch growth performance (Uni and Ferket 2004). With the *in-ovo* injection method, nutritional substances such as carbohydrates, amino acids, vitamins and minerals can be administered into the egg (Aygun 2016, Genc et al. 2019). It is reported that *in-ovo* injection improves growth performance, hatchability, gastro intestinal tract and growth performance (Salmanzadeh et al. 2014, AL-Jomaily and Taha 2020). Poultry embryos are susceptible to oxidative stress due to the high content of polyunsaturated fatty acids in their tissues (Surai et al. 1996 and Foye et al. 2006).

Chrysin (CR), chemical name 5,7-dihydroxyflavone, is one of the flavonoids and is present throughout numerous plant extracts, honey and propolis (Hanedan et al. 2018). In vitro and in vivo studies have demonstrated that CR has neuroprotective (Angelopoulou et al. 2020), hepatoprotective (Temel et al. 2020), antidiabetic (Taslimi et al. 2019), cardioprotective (Farkhondeh et al. 2019), nephroprotective (Temel et al. 2020), anti-inflammatory and antioxidant properties (Kandemir et al. 2017). The aim of this study was to determine the relationship between testicular histology, oxidative stress and sperm quality in *in-ovo* CR treatment in Japanese quails.

Materials and Methods

The study used 720 eggs from the parents of Japanese quail (*Coturnix japonica*) aged 16-20 weeks who were fed a diet comprising 20% crude protein and 2900 kcal/kg metabolizable energy. The eggs were evaluated for shell strength, shape index, size and other characteristics, and those with poor hatchability were removed, while the remaining eggs were randomly divided into four groups. The eggs were incubated for 15 days at 37.5°C ± 0.3°C and 65±5% relative humidity in a pre-development machine that rotated 90 degrees forwards and backwards at 45 degrees from the vertical axis once every hour. Before the final three days of incubation, the following in-ovo injection techniques were carried out. Permission was obtained from Atatürk University Animal Experiments Local Ethics Committee (Protocol no:2024/02-41, Date:26.02.2024).

Control: 0.1 mL physiological saline solution.

CR 25: 0.25 mg Chrysin in 0.1 mL physiological saline solution /egg.

CR 50: 0.50 mg Chrysin in 0.1 mL physiological saline solution /egg.

CR 75: 0.75 mg Chrysin in 0.1 mL physiological saline solution /egg.

The CR dosages were dissolved in saline solution to a final amount of 1 mL. The eggs were sterilized with 70% ethanol, and their flat end (air cell) was perforated. Prior to incubation, the solutions were manually injected at a depth of around 5 mm with a 26 G syringe. Each egg was injected with 0.1 mL of dissolved solution. Following injection, the holes were filled with nail paint and disinfected with 70% ethanol. After injection, the eggs were incubated at 37.5°C ± 0.3°C and 75±5% relative humidity, without rotation. At 60 days after hatching, thirty-two male quails were beheaded using medium sevoflurane anesthesia. The testicular tissues and epididymis were removed, and the right testicular tissue was stored at -20°C for biochemical investigations, while the left testicular tissue was stored in a 10% formaldehyde solution for pathological examination. The epididymis tissue was trimmed in 100 µL of saline solution and the resulting fluid was used for semen analysis.

Total antioxidant status (TAS)

TAS levels were measured using commercially available kits (Relassay, Turkey). The novel automated method is based on the bleaching of characteristic color of a more stable ABTS (2,2′ - Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radical cation by antioxidants. The assay has excellent precision values, which are lower than 3%. The test is calibrated with Standard solution, traditionally know as Trolox Equivalent, a vitamin E analog. The results were expressed as mmol Trolox equivalent/L (Erel 2004).

Total oxidant status (TOS)

TOS levels were measured using commercially available kits (Relassay, Turkey). In the new method, oxidants present in the sample oxidized the ferrous ion-o-dianisidine complex to ferric ion. The oxidation reaction was enhanced by glycerol molecules abundantly present in the reaction medium. The ferric ion produced a colored complex with xylenol orange in an acidic medium. The color intensity, which could be measured spectrophotometrically, was related to the total amount of oxidant molecules present in the sample. The assay was calibrated with hydrogen peroxide and the results were expressed in terms of micromolar hydrogen peroxide equivalent per liter (µm H_2O_2 equivalent/L) (Erel 2005).

Table 1. Reproductive parameters in Japanese quails.

Groups	Total Testis Weight (mg)	Motility (%)	Density (10^6/g)	TAS (µmol/ H_2O_2 Equiv/L)	TOS (µmol/ H_2O_2 Equiv/L)
Control	7069.00±912.13	54.97±2.80^b	59.01±9.89	0.35±0.08^a	35.33±4.18
CR 25	7512.00±1362.87	59.35±3.22^b	59.33±13.17	0.40±0.05^{ab}	33.38±3.48
CR 50	6165.16±1653.89	68.03±5.01^a	54.50±6.92	0.52±0.07^b	26.54±8.94
CR 75	6169.67±1540.85	58.85±4.55^b	53.33±11.27	0.41±0.10^{ab}	29.35±4.92

Superscript letters (a.b) indicate the difference between groups, $p<0.001$.

Testicular weights and semen quality assessment

The testicular tissue of the sacrificed quails was first removed, washed with saline, and weighed on a precision balance (Radwag, R2, Germany). Epididymis tissues were then removed and trimmed in 100 µL saline at 35°C. Twenty µL of the trimmed semen sample was taken and examined in a light microscope (Zeiss, Germany) equipped with a heating table at 35°C at X100 magnification. For each sample, three different microscope fields were examined, and the result obtained was expressed as a percentage.

For density determination in trimmed semen samples, 5 µL of semen sample was added to 995 µL nigrosine dye in an Eppendorf tube and vortexed. 10 µL of the mixture was placed on a Thoma slide and examined with a light microscope at X400 magnification. The number of spermatozoa in the counting area on the Thoma slide was expressed as epididymal sperm density.

Histopathological analysis

At the end of the experiment, the testicular tissues of the quails sacrificed under anesthesia were placed in 10% neutral formaldehyde solution and the testicular tissues were fixed for 72 hours by refreshing the solution every 24 hours. The testicular tissues were then passed through graded ethanol series and xylol series and embedded in paraffin blocks. 5µ thick sections were then taken with a microtome (Leica RM2125 RTS) for histological evaluations. These sections were stained using Mallory's Triple Staining method modified by Crossman for histological examinations.

Testicular tissues on slides were kept in an oven at 60°C for 1 hour to remove the paraffin and stabilize the tissue. For complete removal of the remaining paraffin, the slides were kept in the first xylol for 15 minutes and in the second xylol for 10 minutes. To remove xylol from the tissue and prepare it for water, the sections were placed in a series of decreasing alcohol (absolute alcohol-1, absolute alcohol-2, 96% alcohol, 80% alcohol, 70% alcohol) for 5 minutes each. The sections were then washed in distilled water for 5 minutes to remove the alcohol. After pretreatment for staining, tissues on polylysine slides were kept in hematoxylin solution for 3 minutes for nuclei staining and then washed with distilled water for 5 minutes to remove excess stain. The preparations were then kept in sodium thiosulfate solution for 2 minutes and washed with distilled water. In order to stain the cytoplasm, the preparations were kept in acid fuxin for 1 minute, washed with distilled water and then kept in phosphotungustic acid for 5 minutes to whiten the connective tissue. The sections were washed again with distilled water and kept in aniline blue dye solution for 30 seconds to stain the connective tissue. After washing with distilled water, the sections were soaked in 96% alcohol for 5 minutes, in absolute alcohol twice for 5 minutes each, then in xylol 3 times for 5 minutes each and covered with coverslip and entellan.

A light microscope (Zeiss AXIO Scope.A1) with computer and camera attachment was used for microscopic examination (Çeribaşı et al. 2020).

Statistical analysis

Mean and standard deviation values were calculated for the evaluation of testicular tissue histology, biochemical parameters and reproductive parameters. Values were expressed as mean ± SD (n=8) and analysed by one-way ANOVA followed by Tukey's test. Asterisk (*: $p<0.05$; ns: not significant) indicates statistically significant differences between groups.

Results

Oxidant/antioxidant status and sperm quality assessment results

Testicular weight, epididymal sperm density, total motility, TAS and TOS ratios of the experimental groups are shown in Table 1. There was no statistically significant difference between the groups in terms of total testicular weight and epididymal sperm density. In the CR 50 group, the total motility value was statistically significantly higher compared to the other experimental groups ($p<0.001$). The highest TAS value was

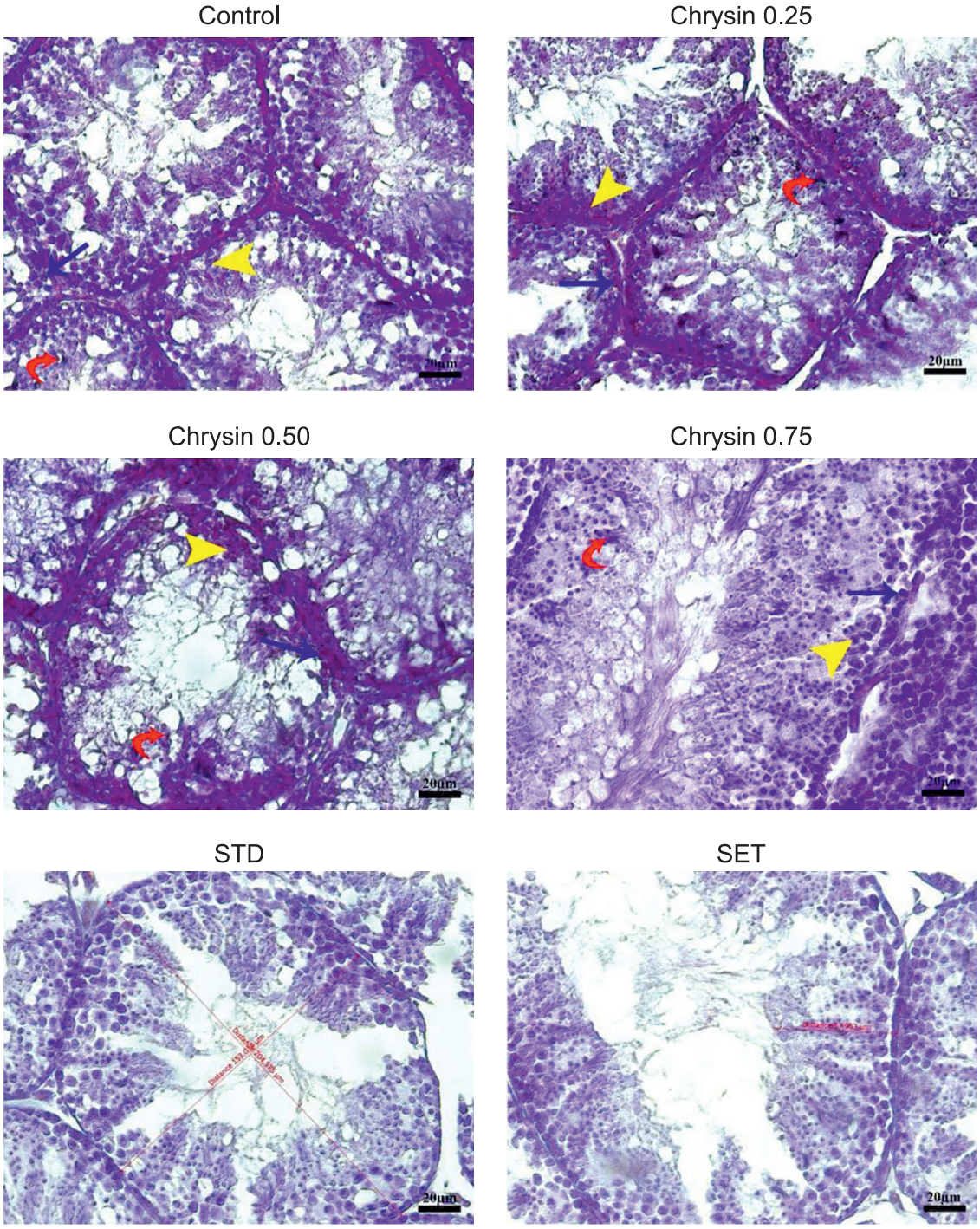

Fig. 1. Testicular tissue micrographs of all bird groups (Mallory's Triple Staining Modified by Crossman, Magnification x400) Blue arrow: spermatogonium; yellow arrowhead: primary spermatocyte; red curved arrow: elongating spermatid; STD: diameter measurement in seminiferous tubule; SET: epithelial thickness measurement in seminiferous tubule.

observed in the CR 50 group, with a statistically significant difference between it and the control group ($p<0.001$). No statistical difference was observed between the groups in terms of TOS value.

Histological findings

In the histological examination of testicular tissues, no significant difference was found in the histological scoring of Control and CR 25 groups ($p>0.05$). Histological scoring of CR 50 and CR 75 groups was significantly higher than Control group ($p<0.05$). There was no significant difference between CR 50 and CR 75 groups ($p>0.05$). Testicular tissue histological images of all groups are shown in Fig. 1 and histological score values are shown in Fig. 2.

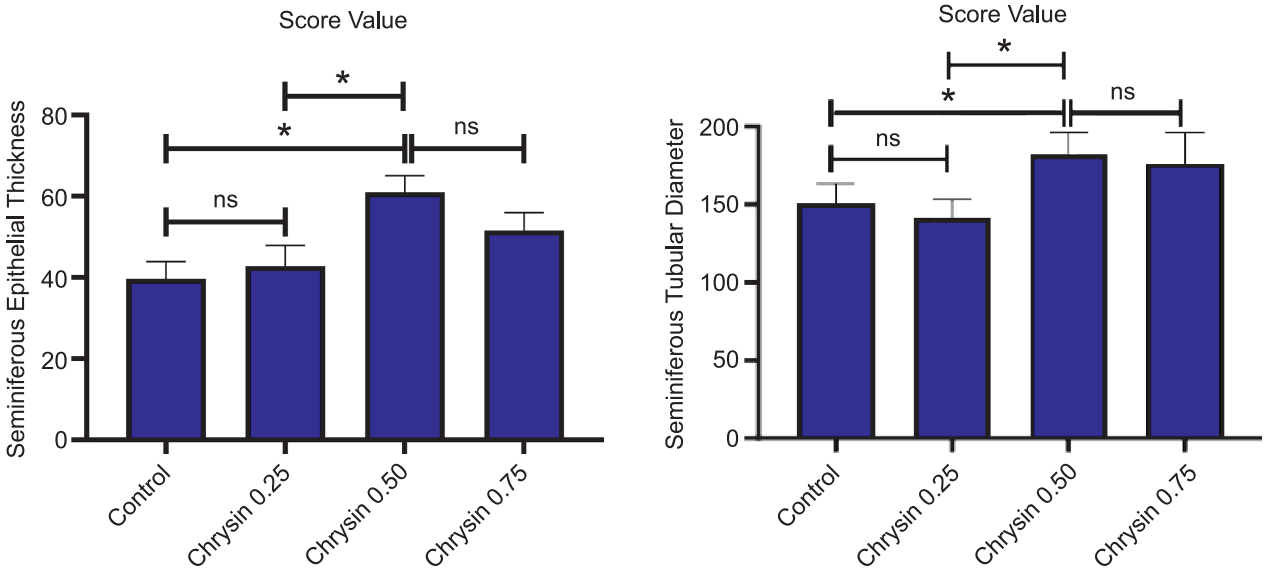

Fig. 2. Evaluation of testicular tissue histology in Japanese quails. Values are given as mean ± SD and analyzed by one-way ANOVA followed by Tukey test. Asterisk (*: $p<0.05$; ns: not significant) indicates statistically significant differences between groups.

Discussion

Japanese quails are widely used in experimental work (Huss et al. 2008). For this reason, it is important to increase the production capacity of Japanese quail and to continue its generation. In the present study, the effects of *in-ovo* CR injection on testicular histology, oxidant status and epididymal sperm quality after puberty were investigated.

In previous studies, it was reported that different compounds injected into Japanese quail eggs had positive effects on basic parameters such as growth performance and hatchability (Kermanshahi et al. 2017a, Kermanshahi et al. 2017b, Hajati et al. 2021, Cerutti et al. 2022). To the best of our knowledge, no study has examined the effects on testicular histology, oxidant status and sperm quality after *in-ovo* injection in Japanese quail. In a study, the effects of androgenic chemicals on different tissues in Japanese quail were investigated, but it was pointed out that cloacal glands may be a marker (Utsumi and Yoshimura 2009). Our study focused on the testis and epididymis and is related to sperm quality.

Basic morphometric characteristics of the reproductive organs are used to measure and predict not only sperm production but also the fertilizing and sperm storage capacity of the breeding male (Akintunde et al. 2021). During the maturation phase, rapid physical growth has an impact on reproductive organ development (Sarabia et al. 2013). The effects of various dietary substances (toxic or non-toxic) on sperm and testis of Japanese quails have been reported in different studies (Ibrahim et al. 2021, Akintunde et al. 2023). In our study, no difference was observed between the experimental groups in terms of testicular weights and epididymal spermatozoon density. This is thought to be due to the *in-ovo* injection dose of CR between 0.25-0.75 mg. However, total sperm motility was statistically significantly higher in the CR 50 group compared to the other experimental groups ($p<0.001$). This suggests that 0.50 mg is the dose of CR that will keep the oxidative stress at the optimum level physiologically.

Low TAS levels are an indication that cells are vulnerable to free radicals. High TOS levels indicate that free radicals are have increased in the body and antioxidant defense systems are insufficient (Mutlu et al. 2024). Previous studies show that CR has antioxidant properties and enhances the antioxidant defense system in tissues (Taslimi et al. 2019, Ileriturk et al. 2021, Kucukler et al. 2021, Kazaz et al. 2022, Demir et al. 2023). In the present study, a numerical increase in TAS and a numerical decrease in TOS levels were observed in CR groups compared to the control group ($p<0.001$). The increase in total motility in the CR 50 group was interpreted as a result of the positive effect of CR on TAS and TOS levels.

Improvement in the thickness and area of the germinal layer in the seminiferous tubules is one of the reasons for improved sperm quality (El-Kazaz et al. 2020). In the present study, thickening of seminiferous tubules in CR groups compared to the control group is remarkable. Especially in the CR 50 group, testicular histoarchitectures improved. In this case, it suggests that it is due to exceeding the physiological oxidative stress level in the CR 75 group ($p<0.05$).

Conclusion

In conclusion, *in-ovo* injection of CR between 0.25-0.75 mg into Japanese quail eggs resulted in an increase in testicular histology score and improvement in antioxidant status. In the CR 50 group, sperm motility resulted in a significant increase in testicular score.

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