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Morphological and morphometric adaptations of testes in broilers induced by glucocorticoid

Rafiqul Islam[1], Nasrin Sultana[1], Ummay Ayman[1], Antora Akter[2], Marzia Afrose[3], Ziaul Haque[1]*

[1]Department of Anatomy and Histology, Bangladesh Agricultural University, Mymensingh, Bangladesh
[2]Department of Surgery and Obstetrics, Bangladesh Agricultural University, Mymensingh, Bangladesh
[3]Department of Livestock Science and Veterinary Medicine, Bangabandhu Sheikh Mujibur Rahman Science and Technology University, Gopalgonj, Bangladesh

**Corresponding author: nsultana.bau@gmail.com, nasrin.sultana@bau.edu.bd*

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Abstract: Glucocorticoids (GCs) cause excess fat accumulation, which leads to fertility dysfunction in broilers. The study investigated alterations in the morphology and morphometry of the testes of broilers in response to GC and dexamethasone (DEX). Male day-old chicks were randomly divided into a control group and three experimental groups (E1, E2, and E3). The control group was fed a commercial broiler ration. The experimental groups were fed a commercial broiler ration containing GC (i.e. DEX 3, 5, and 7 mg/kg, respectively). The testes were collected and stained with haematoxylin and eosin to count the number of testicular seminiferous tubules. An increase in the seminiferous tubule count was initially seen, which declined as both the age of the broilers and the dose of DEX increased. Morphometric measurements, i.e., the testicular capsule thickness, seminiferous tubule diameter, and seminiferous epithelium height, were performed. The initial thickening of the testicular capsule was evident. There was a depletion of the interstitial (Leydig) cell population in the experimental groups with the age and increased with the dose advancement. The diameter of the seminiferous tubules and testicular capsule thickness remained upregulated in the treatment groups with the increased dose of DEX. The initial height of the seminiferous epithelium increased in the experimental groups of broilers. The study suggests that DEX greatly alters the morphological architecture of broiler testes; as a result, it could be said that DEX has the effect on the infertility of the broiler by affecting the morphology as well as the functionality of the testes.

Keywords: biometry; dexamethasone; histoarchitectures; testicles

The poultry sector is an important avenue in increasing the agricultural growth and reducing malnutrition for the people of Bangladesh (Begum et al. 2011). To fulfil the protein demand of the growing human population, broiler production has seen a significant increase in the last few decades. It contributes a major share of the farming system and plays a significant role in fulfilling the per capita meat consumption (Chowdhury 2011). Around 8 trillion broilers are produced worldwide each year and had the fastest growth rate (25%) among all farm animals from 2007 to 2017. Various

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drugs like glucocorticoid (GC) growth promoters (GPs) are frequently added to the broiler feed to maximise the genetic potentiality, to increase the feed conversion ratio (FCR), to promote the survival rate, and to decrease the fatality in birds (Mostafa et al. 2016). Most of these drugs possess deleterious side effects on different organs of the body. Corticosteroids, including GCs, are laboratory-synthesised or naturally produced hormones which can exert side effects at a broad range of doses depending on the route of the administration (Neilsen and Kaye 2014). They are one of the most prescribed drugs worldwide that induce stress conditions that lead to a decreased body weight (Afrose et al. 2018) in broilers. They are also among the most misused drugs that reportedly exert unavoidable and irreparable detrimental effects in animal bodies (Oray et al. 2016).

Male reproductive disorders resulting from endocrine disruptors have recently raised concerns. The parenteral administration of DEX alters the histological architecture in albino rat testes (Abo-Youssef et al. 2018). It also contributes to the expression of proapoptotic proteins that cause testicular germ cell apoptosis in mice (Mahmoud et al. 2009). GCs bind to the GC receptors in the testicular peritubular cells, which play a significant role in sperm transportation (Welter et al. 2020). Testes perform a key role in male fertility with their dual functions of steroidogenesis and spermatogenesis, controlled by a high concentration of testosterone, secreted from Leydig cells (Ing et al. 2014). Exogenous GC leads to irreversible damage at different stages of spermatogenesis immediately after exposure and becomes more incursive if continued for a long time (Hameed et al. 2020). Testosterone has a deep relationship with each step of sexual response in males and low serum testosterone levels, leading to sexual dysfunction (Rastrelli et al. 2018). GC primarily targets the testes by affecting the interstitial (Leydig) cells to control the testosterone production (Hardy et al. 2005). DEX induces oxidative stress which causes disorder in the lipid and glucose metabolism (Lv et al. 2018). Oxidative stress also leads to hyperinsulinemia and increased corticosteroid levels, responsible for excessive fat deposition (Jiang et al. 2008). Fat deposition correlates with the reproductive traits of poultry (Li et al. 2016).

Many studies have been undertaken to study the effect of GCs, especially in the rat model. Mostafa et al. (2016), studied the effects of dietary growth promoter supplementation on the performance, carcass traits, and blood parameters of broiler chicks. The physiological changes in broiler chickens in response to the dietary DEX supplementation have been described by Afrose et al. (2018). The effects of the dietary DEX on immune organs (Sultana et al. 2020a) and the liver of broiler chickens (Sultana et al. 2020b) have also been studied. However, very little information is available regarding the effects of exogenous dietary GC on the histomorphology and morphometry of broiler testes. Therefore, the primary goal of this current study is to investigate the possible dose and age-dependent effects of DEX on the histomorphology and morphometry of broiler testes.

MATERIAL AND METHODS

Ethical approval

The entire study was carried out in the Department of Anatomy and Histology, the Faculty of Veterinary Science, Bangladesh Agricultural University, Bangladesh. The poultry rearing and all the other experimental procedures during this study were performed following the guidelines for the care and use of animals as established by the Animal Welfare and Experimentation Ethics Committee, Bangladesh Agricultural University, Mymensingh [AWEEC/BAU/2021(3)].

Experimental design

Sixty male day-old chicks (DOCs, Cobb-500) were purchased from a commercial hatchery (Provita Hatchery Ltd., Mymensingh, Bangladesh). The DOCs were then randomly divided into four groups, i.e., one control group (C) and three experimental or treated groups, as experimental group 1 (E1), experimental group 2 (E2), and experimental group 3 (E3). The control group, C (n = 20), was fed (*ad libitum*) commercial broiler-type rations (Nourish Poultry and Hatchery Ltd., Dhaka, Bangladesh). The experimental groups – E1, E2, E3 (n = 20 for each) were fed (*ad libitum*) commercial broiler type rations containing corticosteroid (Dexamethasone, BP 0.5 mg; Opsonin Ltd., Dhaka, Bangladesh) at the rate of 3, 5, and 7 mg/kg, respectively.

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Housing, management and feeding

The individual group of birds were housed into separate cages (5 × 4 square feet). The birds were reared on chick paper for the first three days and for the rest of the experiment period, the birds were reared on a deep litter system of housing using sawdust with the provision of artificial light. The rearing shed was properly ventilated to eliminate the probability of hypoxic stress. The brooding temperature was maintained at 35 °C for the first three days. Then the temperature was decreased gradually to 21 °C to rule out heat-induced stress. The relative humidity was maintained at 50–60% throughout the experiment period. The lighting programme was scheduled as: the first week [23 h L : 1 h D (L = light, D = dark)], the second week (16 h L : 8 h D), the third week (16 h L : 3 h D : 2 h L : 3 h D) and the fourth week (16 h L : 2 h D : 4 h L : 2 h D).

The commercial broiler feed was devoid of any antibiotics or growth promoters. The DEX, in a fine powder, was mixed homogenously with the feed at the mentioned dosage for the respective groups. Special care and monitoring were ensured so that each bird of the treatment groups received the desired amount of DEX with the feed. The starter and grower feed composition (Sultana et al. 2020b) and pellet size were chosen to meet the recommended nutrient requirements. The birds were fed the starter feed for the first 14 days and then shifted to the grower feed for the rest of the experimental period. An adequate amount of fresh drinking water was available *ad libitum.*

The feeders and drinkers were cleaned daily, whereas the old litter was replaced with fresh litter on every alternate day.

Sample collection

To study the effects of DEX on the reproductive organs of the male broilers, five experimental birds from each group were sacrificed by the manual cervical subluxation method and dissected immediately after slaughtering. The testes in broilers are located deep in the abdominal cavity. Therefore, the abdominal organs, particularly the intestinal parts were removed to make the testes visible. Then, the narrow jaws of the forceps were placed carefully below the left testicle, located on the left side of the midline in the dorsal coelom, near the caudal end of the left lung and the cranial end of the left kidney, which was then gently pulled out. Thus, samples were collected on days 14, 21, and 28 from each group (except for E1 on day 28). After collection, the tissue samples were washed with physiological saline (0.9%) and preserved in 10% buffered formalin. Then, the samples were kept for routine staining procedures and for the histological study.

Gross morphology

For this, the gross characteristics, i.e. anatomical location, colour, size, and shape, were examined during the sample collection.

Histomorphological study

For the histological investigation, the testicular tissue samples were dehydrated using an ascending graded alcohol followed by clearing in three changes of xylene. The tissue infiltration was undertaken with different grades of melted paraffin (49, 55 and 58 °C) at 30-min intervals. Then the tissue samples were embedded with melted paraffin (58 °C). Finally, 6 µm thick slices of tissue sections were obtained by using a sliding microtome (MIC 509; Euromex, Tokyo, Japan). The tissue sections were stretched in a floatation bath (37 °C) and mounted on an adhesive (50% egg albumin and 50% glycerine) coated frosted glass slide. Then the slides were dried on a slide warmer at 37 °C. The tissue sections were then processed and stained with Mayer's haematoxylin and eosin (H&E) stain. All the stained tissue sections were examined by one person to avoid any kind of individual variation and analysed blindly to avoid any bias. Five randomly selected focuses from each tissue section were studied at × 40 magnification for counting the number of seminiferous tubules. Only the seminiferous tubules that were intact and completely located within the focus were counted.

Morphometric study

The thickness of the capsule, the diameter of the seminiferous tubules, and the height of the seminiferous epithelium were considered for the morphometric measurements, which were performed using

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a calibrated stage micrometre in µm (micrometres). Five focuses were selected randomly from each section, and the mean value was calculated. Thus, a total of ten sections were morphometrically evaluated from each group.

Photomicrographs

The necessary pictures were taken by a photomicroscope (U-LH50HG; Olympus Corporation, Tokyo, Japan) at × 40 and × 100 magnification to better illustrate the obtained results. For each section, ten randomly selected fields from each slide were captured to evaluate the testicular integrity of each group of broilers.

Statistical analyses

The biometric measurement results were analysed using SPSS software (IBM SPSS Statistics v22, USA). The Shapiro-Wilk test evaluated the normality of the data set. In all the trials, the data were expressed as the mean ± standard error of the mean (SEM). Levene's test was performed to check the equality of the variance and the differences among the groups of birds were compared using a one-way analysis of variance (ANOVA) with post hoc Duncan's multiple range test where $P < 0.05$ was considered significant and $P < 0.001$ was considered highly significant.

RESULTS

Gross anatomical alterations

No gross visual anatomical changes were observed in the testes of the DEX treated groups (Figure 1). The testes were found in their original anatomical location in all the broilers. A uniform colour and size with a regular shape were seen both in control group and treated groups of broilers.

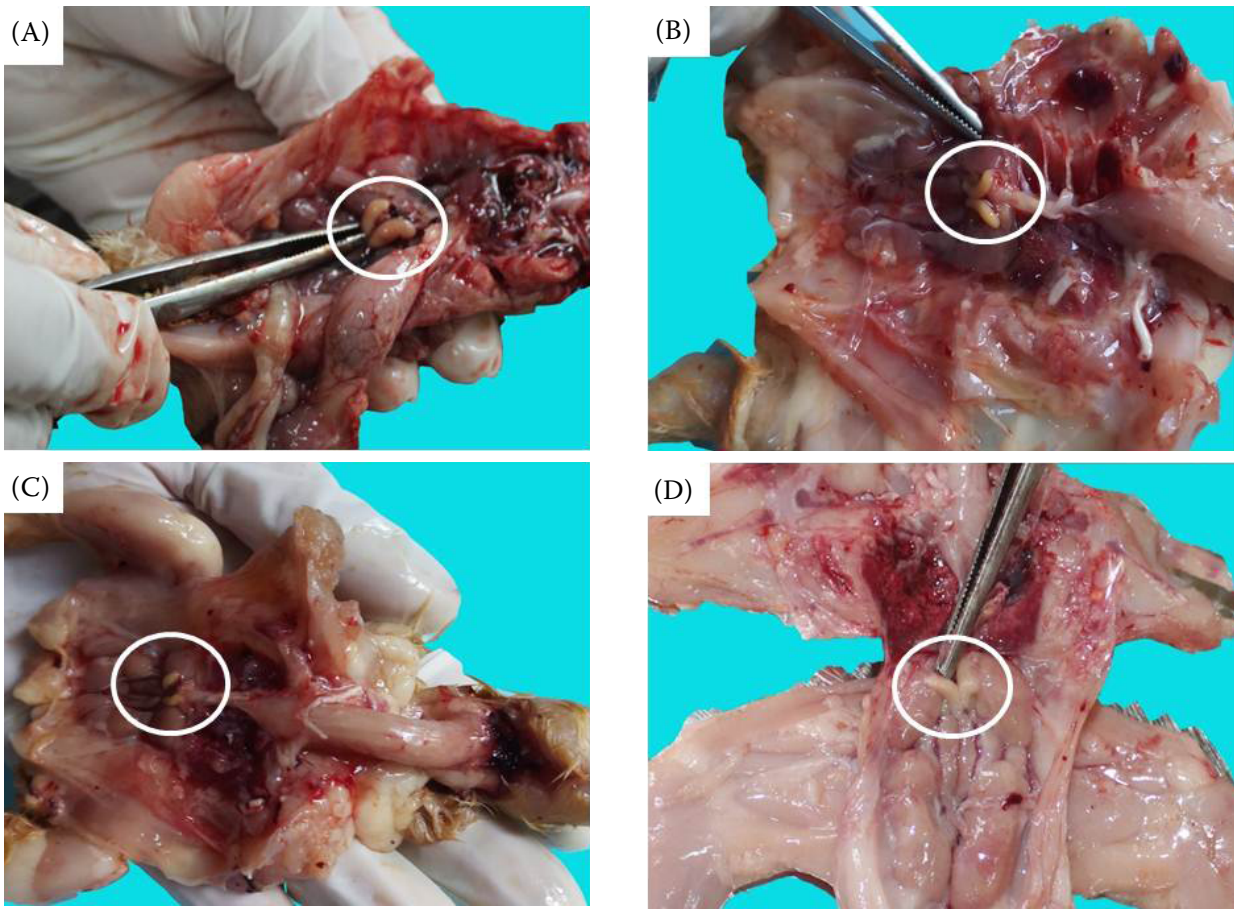

Figure 1. Gross view of the broiler testes during the collection of the sample on day 14
(A) Control group. (B) Experimental group 1, E1. (C) Experimental group 2, E2. (D) Experimental group 3, E3

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Histomorphological alterations

In the control group, the testes were covered with a thick, dense, fibrous tissue capsule, tunica albuginea. A single layer of flattened squamous epithelium with an elongated nucleus was observed on the free surface of the tunica albuginea. Blood vessels were found in the tunica vasculosa just beneath the tunica albuginea (Figure 2A1–A2). A regular and undisturbed pattern of fine oval or twisted-shaped seminiferous tubules was observed within the testes. The seminiferous tubules were enclosed in a very thin layer of collagen fibres. A seminiferous epithelium containing Sertoli cells was found lying in close contact with the basal membrane of the tubule. The centres of the tubules were filled with apical cytoplasm of the columnar sustentacular (Sertoli) cells. Between the sustentacular cells, germ cells were located extending up to the lumen of the tubules (Figure 3A1–A2). Single or small clusters of interstitial (Leydig) cells containing a small, round, and centrally located nucleus and blood vessels were found in the interstitial tissue of the control group broilers. No distinct connective tissue septa were observed.

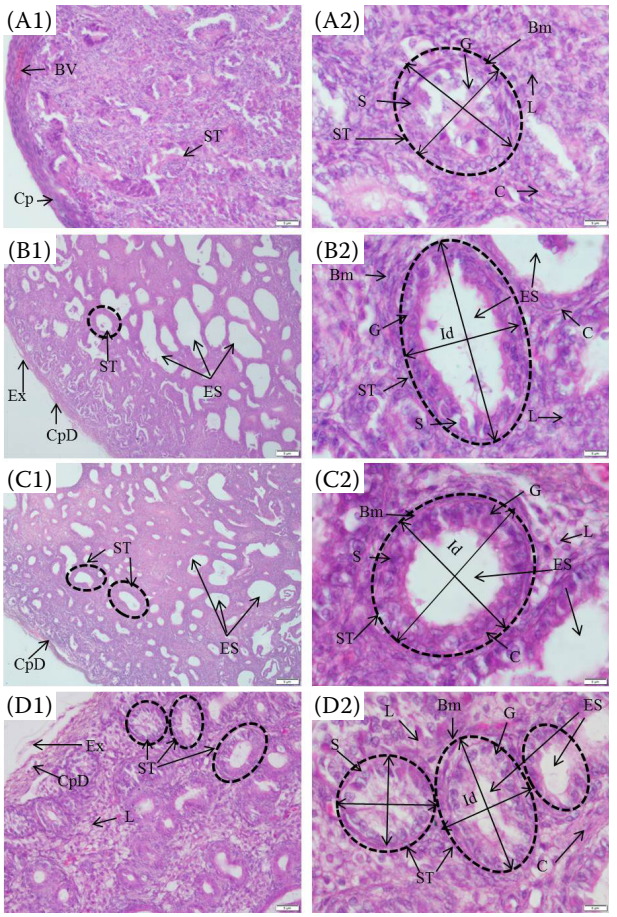

Figure 2. Representative photomicrographs of the transverse section (H&E stained) of the testis in the 21 day-old control broiler (A1, A2), experimental group 1 (B1, B2), experimental group 2 (C1, C2) and experimental group 3 (D1, D2) at 40 × and 100 × magnification

Bm = basal membrane; BV = blood vessel; C = collagen fibre; Cp = capsule; CpD = decreased capsular thickness; ES = empty spaces within the seminiferous tubule; Ex = exfoliation of squamous cell layer from the free surface of the testicular capsule; G = germ cell; Id = increased seminiferous tubule diameter; L = Leydig cell; S = Sertoli cell; ST = seminiferous tubule

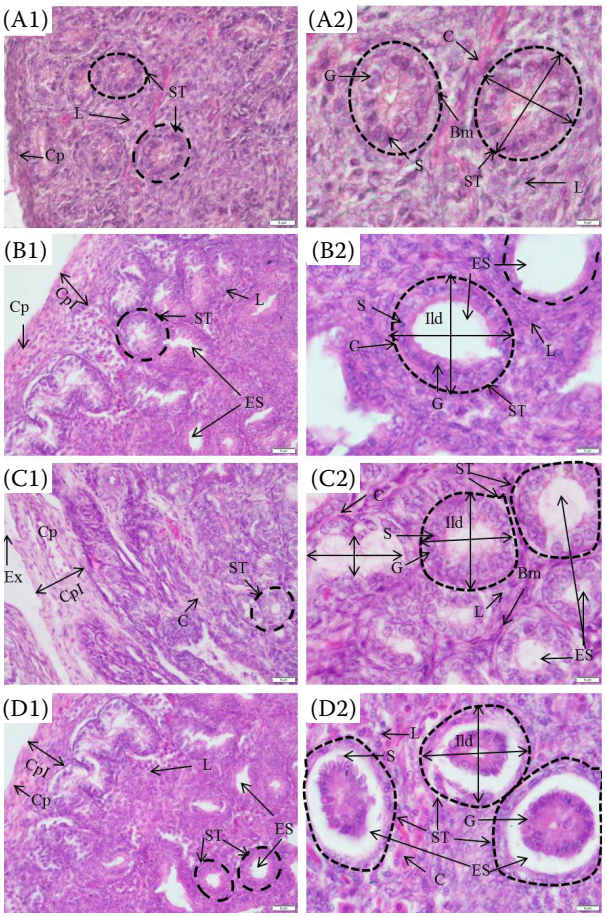

Figure 3. Representative photomicrographs of the transverse section (H&E stained) of the testis in the 14-day old control broiler (A1, A2), experimental group 1 (B1, B2), experimental group 2 (C1, C2), and experimental group 3 (D1, D2) at 40 × and 100 × magnification

Bm = basal membrane; C = collagen fibre; Cp = capsule; CpI = increased capsular thickness; ES = empty spaces within the seminiferous tubule; Ex = exfoliation of squamous cell layer from the free surface of the testicular capsule; G = germ cell; Ild = increased the smallest diameter of seminiferous tubule; L = Leydig cell; S = Sertoli cell; ST = seminiferous tubule

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On day 14, the testes of the DEX-treated birds revealed a denser and thicker fibrous capsule compared to the control group. However, exfoliation of the squamous cell layer was seen in the E2 group, which was found to be more evident in the E3 group. There was a slightly increased amount of collagen fibres surrounding the seminiferous tubules. The number of seminiferous tubules was significantly increased in each group, but the increase was highly significant ($P < 0.001$) in the E2 group when compared to the control group (Figure 4). In the E1 group, the seminiferous tubules were found with the usual morphological appearance in the peripheral region, but more distorted, disorganised seminiferous tubules were found along with creating irregular empty spaces due to sloughing off some portions towards the centre. The tubular diameter was slightly increased. The enlargement and increase in the germ cell population in the seminiferous tubules was obvious in the E1 and E2 groups, but decreased in the E3 group. The E2 group of birds showed a very compact arrangement of seminiferous tubules, whereas the mass of seminiferous epithelium was found in the centre of the tubules developing a wide space around them in the E3 group (Figure 3).

On day 21, the squamous cell layer was diminished in the DEX-treated broilers. The capsular thickness was also reduced. An increased number of collagen fibres were seen in the interstitial tissue, which was more evident in the E2 and E3 groups. The numbers of seminiferous tubule were numerically decreased in comparison to the control group which was not statistically significant. The germ cell population decreased when compared to day 14. The Leydig cell count was also decreased, creating empty spaces around the seminiferous tubules (Figure 2).

On day 28, this epithelial layer was found completely sloughed off from the surface of the tunica albuginea, reducing the testicular capsule's thickness. The thickness of the testicular capsule was also decreased. The depletion of both the sustentacular (Sertoli) cell and germ cell population along with the disruption of the regular pattern of these cells in the seminiferous tubule was seen. This change was found more evident with an increase in both doses of DEX and with the age of the broilers. However, the definite structures of the seminif-

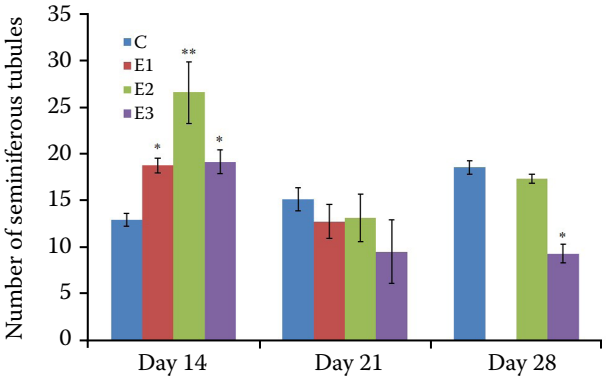

Figure 4. Analysis of the effects of glucocorticoids on the seminiferous tubule count in the DEX treated broilers

The data are expressed as the mean ± standard error and the differences among the groups of birds were compared using a one-way ANOVA with post hoc Duncan's multiple range test. Column with asterisks (*) are significantly different from the control group ($P < 0.05$) and double asterisks (**) are highly significantly different from the control group ($P < 0.001$)

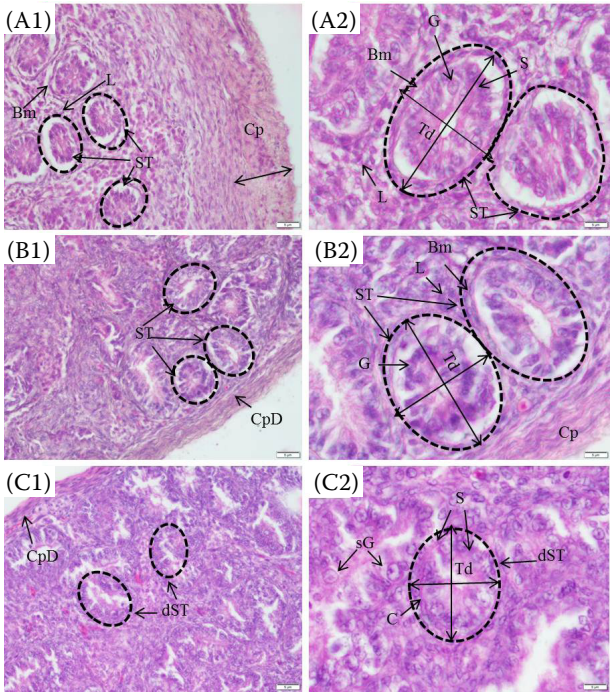

Figure 5. Representative photomicrographs of the transverse section (H&E stained) of the testis in the 28 day-old control broiler (A1, A2), experimental group 2 (B1, B2), experimental group 3 (C1, C2) at 40 × and 100 × magnification

Bm = basal membrane; C = collagen fibre; Cp = capsule; CpD = decreased capsular thickness; dST = distorted seminiferous tubules; ES = empty spaces within the seminiferous tubule; G = germ cell; L = Leydig cell; S = Sertoli cell; sG = germ cells arranged in a scattered way; ST = seminiferous tubule; Td = seminiferous tubule diameter

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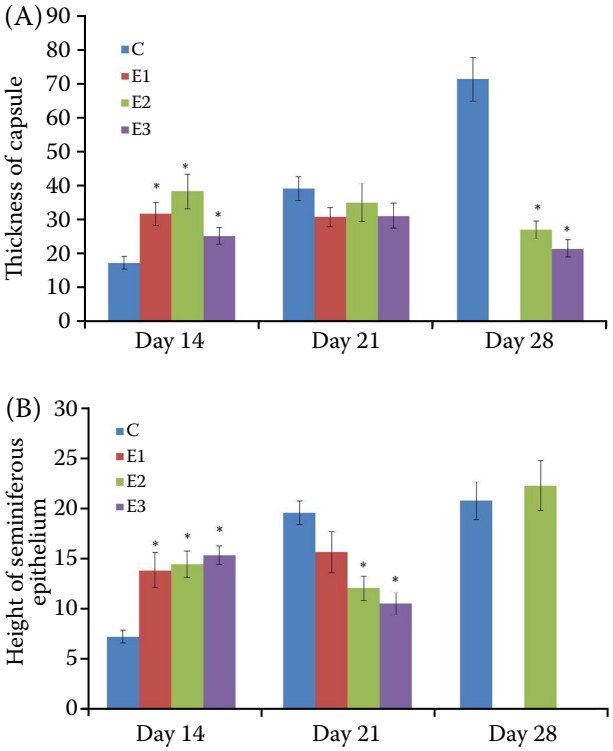

Figure 6. Morphometrical analysis of the effects of glucocorticoids on the DEX treated broilers

(A) Testicular capsule thickness. (B) Height of the seminiferous epithelium when compared to the control group of the respective day. The data are expressed as the mean ± standard error and the differences among the groups of birds were compared using a one-way ANOVA with post hoc Duncan's multiple range test. Column with asterisks (*) are significantly different from the control group ($P < 0.05$)

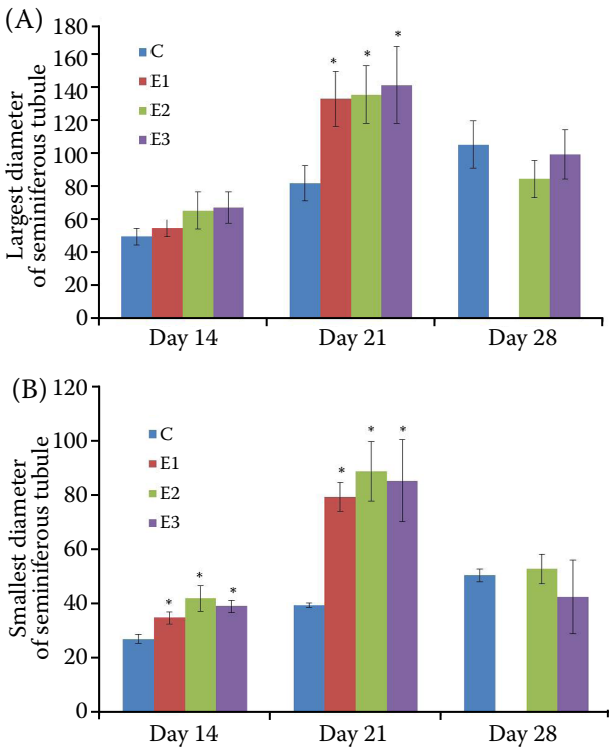

Figure 7. Morphometrical analysis of the effects of glucocorticoids on the DEX treated broilers

(A) Largest diameter. (B) Smallest diameter of the seminiferous tubules when compared to the control group of the respective day. The data are expressed as the mean ± standard error and the differences among the groups of birds were compared using a one-way ANOVA with post hoc Duncan's multiple range test. Column with asterisks (*) are significantly different from the control group ($P < 0.05$)

erous tubules were almost compromised with the derangement of the seminiferous epithelium and distorted basal membranes were found to be disrupted in the E3 group on day 28. This led to the scattered dispersion of the Sertoli cells and germ cells throughout the testes. Nevertheless, the decrease was significant in the E3 group compared to the control group (Figure 4). The Leydig cells were almost depleted and the remaining Leydig cells showed advanced atrophy (Figure 5).

Morphometric alterations

For the morphometric data analyses, the DEX treated groups were compared with the control group on the respective day. The thickness of the capsule was morphometrically measured from both the cranial and caudal pole, and the lateral and medial surface of each testis section. On day 14, the thickness was significantly increased in all the treated groups. However, the E3 group showed a significant decrease in the thickness than the E2 group did (Figure 6A). No significant difference was observed in the largest diameter of the seminiferous tubules, but the smallest diameter was increased significantly. The height of the seminiferous epithelium was also increased significantly (Figure 6B).

On day 21, no significant difference was observed in the testicular thickness from the control group. Both the greatest and lowest diameter seminiferous tubules were significantly increased (Figure 7A–B). The height of the seminiferous epithelium was significantly decreased in the E2 and E3 groups (Figure 6B). On day 28, the capsular thickness showed a significant decrease from the control group (Figure 6A). No significant difference was

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observed in the diameter of the tubules and the height of the seminiferous epithelium. The height of the seminiferous epithelia could not be measured morphometrically because the seminiferous tubules were found to be distorted with a scattered dispersion of germ cells.

DISCUSSION

In this study, three different doses of DEX were fed to broilers to observe the effects of GC on the histomorphology and morphometry of the testes. The testes of the control group broilers showed general histological characteristics as described by Fragoso et al. (2013). However, the treated groups of broilers revealed significant changes in the histological architectures of the testes.

In the gross study, no distinct changes were observed in the four groups. Though a decrease in the seminiferous tubule count with apoptosis of germ cells and the depletion of the Leydig cell population was seen in the histological investigation. However, the findings of Yazawa et al. (2000) justify our results where they described that a DEX treatment does not have any significant effect on the testicular weight. Bonetti et al. (2008) also described testicular hypoplasia due to an anabolic steroid treatment.

On day 14, the fibrous connective tissue and thickness of the testicular capsule were increased in the treatment groups of birds. A similar result was reported in mice treated with ketoconazole (Khayat et al. 2009). The number of seminiferous tubules showed a significant increase though Kheirabad et al. (2015) described a decreased number of seminiferous tubules due to the GC treatment. This increase was highly significant, making the compact arrangement of the seminiferous tubules reasonable in the E2 group. Around the seminiferous tubules, an increased amount of collagen fibres was observed through a study conducted by Welter et al. (2020) who described the expression of the GC receptors by DEX without affecting the collagen expression. Our study also found an increase in the germ cell population in the DEX-treated broilers, which is a novel finding because no other researcher mentioned this type of change as per the author's knowledge. However, we also found the increased thickness of the seminiferous epithelium in treated groups compared to the control group, which may be due to the increased Sertoli cell and germ cell population. The mass of the seminiferous epithelium was found in the centre of the tubules developing a wide space around them in the E3 group, which agrees with Abo-Youssef et al. (2018). However, the diameter of the seminiferous tubules was increased in the treated groups, which is opposite to the findings of Badawy (2018). A significantly reduced lumen diameter and thickness of the germinal epithelium due to stress-mediated GC was described by Kheirabad et al. (2015).

On day 21, the thickness of the testicular capsule decreased. This may be due to the exfoliation of the squamous cell layer from the surface of the free surface of the testicular capsule. Mao et al. (2018) described this kind of exfoliation for testicular germ cells. Both the Sertoli cell and germ cell populations were decreased in the DEX-treated groups. The loss of the germ cell population might be due to the corticosteroid-induced apoptosis of the germ cells, as described by Abo-Youssef et al. (2018). The height of the seminiferous epithelium was also reduced. The apoptosis of the germ cells might be the core reason behind this. Depletion of the Leydig cells with vacuolation and a wider interstitial space was found, which is in agreement with the findings of Hameed et al. (2020).

On day 28, the squamous cell layer was completely lost from the surface of the testicular capsule, and the thickness of the capsule was also reduced. Distortion of the basal membrane of the seminiferous epithelium with a marked depletion of both the Sertoli cell and germ cell populations was evident at day 28, which matches the findings of Hameed et al. (2020) and Kheirabad et al. (2016). This result agrees with Rai et al. (2003) who attributed the loose arrangement and sloughing of these cells. The seminiferous tubules were disorganised and fused with a wide-open empty lumen similar to the findings of Badawy (2018). The number of seminiferous tubules was also decreased significantly in the E3 group, which agrees with the results described by Kheirabad et al. (2015). These three findings (distortion, fusing of seminiferous tubules, and decreased number of tubules) are coherent with each other. A significant depletion of the Leydig cell population was also seen.

Testosterone is produced by Leydig cells. GC promotes the apoptosis of the Leydig cells (Mahmoud et al. 2009) and plays an important role in reducing the blood testosterone levels (Hardy et al. 2005). It also affects the overall growth and haemato-

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logical parameters of broilers (Afrose et al. 2018). GC exerts a direct effect on Sertoli cells and induces retardation (Hameed et al. 2020). Sertoli cells provide nutritional support and fulfil the energy requirements of the germ cells (Crisostomo 2018), and the retardation of these cells leads to impaired testicular development.

On day 14, both the thickness of the testicular capsule and the height of seminiferous epithelium significantly increased in the DEX-treated broilers compared to the control group. The smallest diameter was also increased in treated groups. These means the thickness of the capsule and the the diameter of the seminiferous tubules were increased. On day 21, the height of the seminiferous epithelium significantly decreased, which supports the histological investigation's compatibility. There was an increased diameter of seminiferous tubules, though Badawy (2018) reported a decrease in the diameter due to the GCs. The seminiferous tubule diameter plays a significant role as there is a positive relationship between the tubular diameter and the spermatogenetic activity of the testes (Franca and Russell 1998). On day 28, the thickness of the capsule had shown a significant decrease in the control group. Exfoliation, which was seen in histological observation, might be the reason behind it.

Looking at the obtained data from the present study, it is evident that the DEX treatment affects the histomorphology and morphometry of the testes. The direct effect of the DEX on the Leydig cells leads to a testosterone deficiency, and the Sertoli cell depletion affects the spermatogenesis.

Changes in the histomorphology and morphometry of the testes of broilers due to different doses were studied. The initial thickening of the testicular capsule with a declining thickness later on was observed. The number of seminiferous tubules was increased at 14 days, but decreased at 28 days. Exfoliation of the surface epithelium of the capsule was seen. Increased amounts of germ cells were found on day 14, which decreased with the growing age. The loss of integrity of the seminiferous tubules was evident with the increasing age. The Leydig cell count was decreased and almost depleted on day 28. All the data obtained in this study indicate that exogenous dietary GCs possess hazardous effects on broiler testes, altering the testicular function causing infertility in broiler males. Further studies are recommended to study the effects of GC on the endocrine glands and hormones, semen volume, viable spermatozoa count, and spermatozoa's fertilisation capacity, which are related to fertility and reproduction.

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Conflict of interest

The authors declare no conflict of interest.

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