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Exogenous Testosterone Causes Delayed Postnatal Testicular Development and Disrupts Spermatogenesis

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ABSTRACT

Proper postnatal development of testis is prerequisite for acquisition of full functional potential of testis. The initiation and maintenance of spermatogenesis depend on testosterone, and the production of mature sperm is highly reliant on androgen activity in the testis. The goal of the study was to investigate the effect of exogenous testosterone on testicular biometry and histomorphometry, and seminiferous epithelium in Black Bengal goat during the postnatal development. A total of 42 Black Bengal goats were divided into two categories: control and testosterone-treated; and each group again subdivided into seven groups (n=3) according to age, *viz.* group-I (at birth or day 0; d0), group-II (1 week of age), group-III (2 weeks), group-IV (1 month), group-V (2 months), group-VI (4 months), and group-VII (6 months). Goat kids of testosterone-treated group were injected with testosterone hormone @ 125 mg/kid (0.5 ml of Testanon™ 250 Injection, IM, 250 mg/ml, Nuvista Pharma Ltd., Bangladesh) IM weekly until the sample collection. The testicular development was slow up to 2 weeks of age, and after that, in control goats, a quick growth of the testicles was seen. In contrast, a slow trend of testicular development was continuous throughout the study period (postnatal 6 months) in testosterone-treated goats. A continuous and gradual developmental process of testis was observed in control goats. Seminiferous tubules were shown to have a marked increase in diameter at 4 months indicates initiation of spermatogenesis at this age, and completion of the first wave of spermatogenesis, *i.e.* establishment of spermatogenesis was observed by 6 months of age as confirmed by the presence of all types of cells of spermatogenic lineage in the seminiferous epithelium including spermatozoa were found attached to the Sertoli cells at their ad luminal border, and in the lumen of the seminiferous tubules. Continuous administration of exogenous testosterone exerted a negative effect on the postnatal developmental process of testis of Black Bengal goats. The postnatal developmental process of testis was very slow, and the proliferation of spermatogonia and lumenization of the seminiferous tubules, also known as seminiferous epithelium stratification, were not seen in testosterone-treated goats until they were 6 months old indicates delayed postnatal testicular development and disrupts spermatogenesis.

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Introduction

Proper postnatal development of testis is prerequisite for proper function of testis. Androgens play a well-known role in the growth of male reproductive organs (Patrão *et al*., 2009; Wang *et al*., 2009). Postnatal growth and maturation of testis are controlled through a balance of genetic and androgens, mainly testosterone (Smith and Walker, 2014). Moreover, research on the development of the postnatal testis has made it possible to describe the first spermatogenic wave in animals.

Testosterone is a multifunctional hormone that has a variety of physiological effects on the development of the male reproductive system, including the basic sexual characteristics that include testicular descent, spermatogenesis, and desire induction (Kalfa *et al*., 2019; Patel *et al*., 2019) as well as regulation of secondary male characteristics (Plant and Marshall, 2001). Testosterone, mainly produced in testes by Leydig cells (Abaas, 2020; Eurell and Frappier, 2019), a key hormone required for spermatogenesis (Defalco *et al*., 2013). Changes in testosterone levels throughout postnatal development can have a significant impact on not just the size and weight of the accessory sex organs and testes, but also the spermatogenesis process (Abaas, 2020; Welsh *et al*., 2009). Few research have shown how exogenous androgenic substances affect prostate growth (Wolf *et al*., 2002; Hotchkiss *et al*., 2007b). In addition, in order to treat other male disorders such as cryptorchidism, and testicular cancer, it is crucial to comprehend how exogenous androgens function during particular prenatal programming windows (Welsh *et al*., 2008). Previously the impacts of exogenous testosterone was demonstrated on the biometry and histomorphometry of male accessory sex glands (Gofur *et al*., 2014), and urethra (Gofur, 2019) of black Bengal goats.

Biology of reproduction can be studied using the goat model. The goat testis resembles other domestic animals, rodents, and primates in terms of structure and function. The only breed of goat officially recognized in Bangladesh is the Black Bengal, which occupies a notable position among the genetic resources for goats worldwide (Kabiraj, 2011). The Bangladesh Government still trying to uphold sustainable livelihoods in rural Bangladesh through mass rearing and production of Black Bengal goat. Through selective breeding, Bangladesh Livestock Research Institute (BLRI) has been working to enhance the Black Bengal goat since 1998 (Faruque *et al*., 2010). We hypothesized that exogenous testosterone would delay normal postnatal development and disrupt normal spermatogenesis. However, the effect of exogenous testosterone on postnatal testicular development of Black Bengal goats is not investigated yet. To ascertain the impact of exogenous testosterone on the postnatal testicular development in a Black Bengal goat model, the current study was designed.

Materials and Methods

Animals

Pregnant Black Bengal goats were bought from local markets near the University of Rajshahi. Black Bengal goat kids varying in postnatal age from birth (day 0) to 6 months were reared under standard farm conditions. The testes of these goats were used to observe the effect of exogenous testosterone on the postnatal development of testis of Black Bengal goat. The goats were divided into two categories: control and testosterone-treated, each of which were again subdivided into seven groups according to age (n=3), *viz.* group-I (at birth or day 0; d0), group-II (1 week of age), group-III (2 weeks), group-IV (1 month), group-V (2 months), group-VI (4 months), and group-VII (6 months). To determine the age of the goats under

study, precise birth records were maintained. Animal experiments were executed according to the rules set by the Institutional Animal, Medical Ethics, Biosafety and Biosecurity Committee of the University of Rajshahi, Bangladesh (Memo No. 293(13)/320/IAMEBBC/IBSc).

Testosterone administration

Goat kids of testosterone-treated group were injected with testosterone hormone @ 125 mg/kid (0.5 ml of Testanon™ 250 Injection, 250 mg/ml, Nuvista Pharma Ltd., Bangladesh. Testanon 250 Injection (1 ml) includes testosterone propionate 30 mg, testosterone phenylpropionate 60 mg, testosterone isocaproate 60 mg and testosterone decanoate 100 mg, in 1 ml arachis oil IM weekly until the sample collection.

Sample collection

Male kids/bucks of both groups (control and testosterone-treated) at day 0, 1-, 2-weeks, 1-, 2, 4- and 6-months of ages were slaughtered and the testis were collected. Following that, the right testes were employed for a gross biometrical research whereas the left testes were fixed in a 10% formalin solution for histomorphometrical analysis.

Gross biometrical study

With the aid of a vernier caliper, the general anatomical dimensions (length, breadth) of a collection of growing testes were measured. The findings were recorded against a centimeter scale. A triple beam balance was used to weigh the right testis, and the results were recorded in grams.

Histomorphometrical study

Following the standard histological procedure for paraffin sections, the chosen formalin-fixed samples were treated in the lab. According to Gofur *et al.* (2008), sections were cut at a thickness of 5 µm, and stained for microscopic examination using a standard hematoxylin and eosin stain. Using compound microscopes with magnifications of 10, 20, and 40, the stained slices of postnatally growing testis were carefully examined. A photographic microscope system (Digital camera model: C-B5, OPTIKA, Italy fitted with a microscope, Model B-293PLi, OPTIKA, Italy) was used to capture the photographs of the stained tissue sections.

Statistical analyses

All values were presented as mean±SE. Differences in biometrical and histomorphometrical values among the different postnatal ages were evaluated by one-way analysis of variance (ANOVA), followed by Turkey HSD post-hoc analysis using statistical software available online (https://astatsa.com/One-Way_Anova_with_TukeyHSD/). Differences in biometrical and histomorphometrical values of same age group between control and testosterone-treated groups were assessed by unpaired t-test using statistical software available online (https://www.graphpad.com/quickcalcs/ttest2/). Significant differences were defined as P values of 0.05 or less.

Results and Discussion

Effects of exogenous testosterone on biometry of postnatal developing testis of Black Bengal goat

The testes of control and testosterone-treated Black Bengal goats were measured for length, width, and weight on day 0, one week, two weeks, one, two, four, and six months old to examine the effects of exogenous

testosterone on the testicular biometry during the development after birth. The comparative biometrical values of postnatal developing testis of control and testosterone-treated Black Bengal goats of different age groups are shown in Fig. 1–3. The testicular biometrical values were increased with the increment of age in both control and testosterone-treated goat kids. Different postnatal age groups had distinct testicular biometric parameters. In control goats, the testicular development was slow up to 2 weeks of age evidenced by insignificant biometric values across age groups (d0 vs 1w vs 2w), and after that, a quick development of the testicles was seen, which was demonstrated by a considerable difference in biometric values between age groups (1m vs 2m vs 4m vs 6m) until puberty ($p<0.05$). In contrast, testosterone-treated goats showed a sluggish trend in testicular development over the period under study (up to 6 months postnatal age).

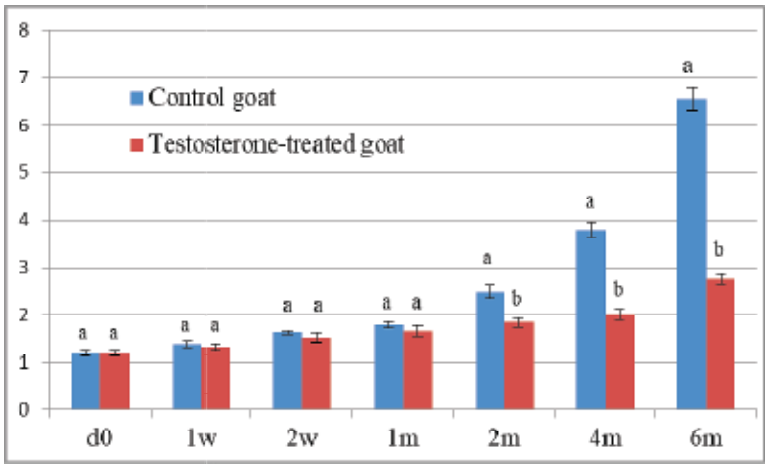

Fig. 1. Comparative values of testicular length between control and testosterone-treated goats. Values in a paired column with different superscripts indicate a significant difference between the control and testosterone-treated goats at a specific postnatal age group ($p<0.05$).

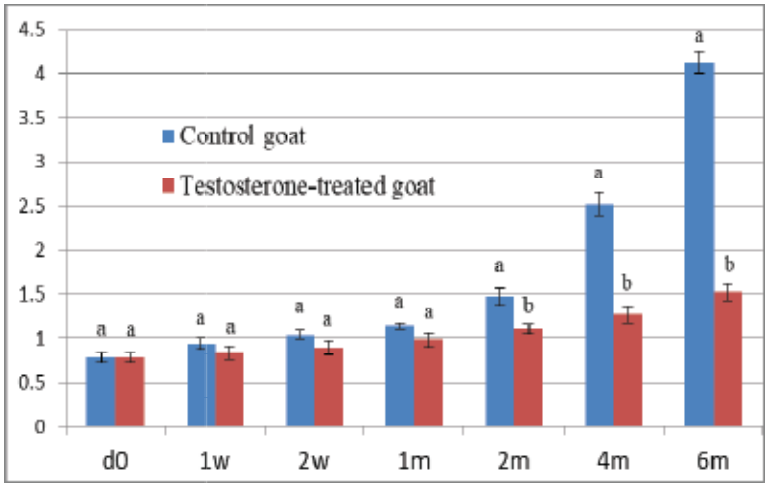

Fig. 2. Comparative values of testicular width between control and testosterone-treated goats. Values in a paired column with different superscripts indicate a significant difference between the control and testosterone-treated goats at a specific postnatal age group ($p<0.05$).

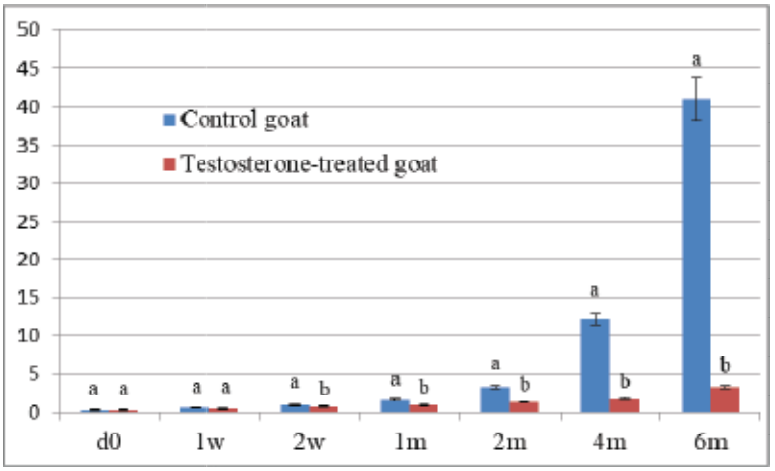

Fig. 3. Comparative values of testicular weight between control and testosterone-treated goats. Significant differences between control and testosterone-treated goats at a particular postnatal age group are indicated by values in paired columns with different superscripts ($p<0.05$).

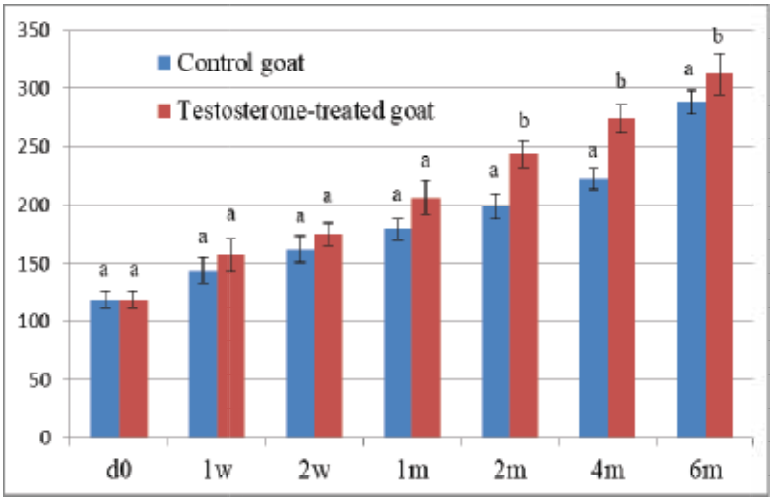

Fig. 4. Comparative values in thickness of tunica albuginea between control and testosterone-treated goats. Significant differences between control and testosterone-treated goats at a particular postnatal age group are indicated by values in paired columns with different superscripts ($p<0.05$).

In comparison at a particular age group between control and testosterone-treated goats, significant variations ($p<0.05$) were detected in testicular length and width at 2 months of age, and in weight at 2 weeks of age and onward (Fig. 1–3). At six months of age (the onset of puberty), the testicular length and breadth were enlarged in control goats by 5–6 times compared to birth, but only by about 2 times in testosterone-treated goats. On the contrary, testosterone-treated goats had testicular weight that was only about 8 times higher at puberty than it was at birth, while testicular weight in control goats was more than 100 times higher at that time.

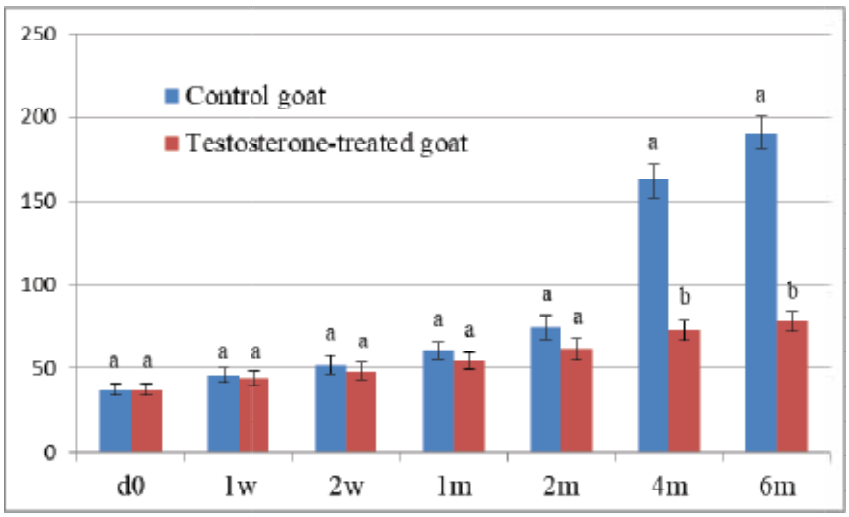

Fig. 5. Comparative values in diameter of seminiferous tubules between control and testosterone-treated goats. Significant differences between control and testosterone-treated goats at a particular postnatal age group are indicated by values in paired columns with different superscripts ($p<0.05$).

Effects of exogenous testosterone on histomorphometry of postnatal developing testis of Black Bengal goat

The histomorphometrical analyses showed the variations in tunica albuginea thickness and seminiferous tubule width at various ages throughout the postnatal development of testis in both groups of Black Bengal goats. With the advancing age of Black Bengal goats, a progressive increase in tunica albuginea thickness was seen during the postnatal development (Fig. 4). Although there were changes in the thickness of tunica albuginea among postnatal age groups, both control and testosterone-treated goats showed significant differences ($p<0.05$) between postnatal age groups starting at 1 month of age and on. On the other hand, from 2 months of age and forward, both in control and testosterone-treated goats, significant variations ($p<0.05$) in the diameter of seminiferous tubules were seen among the postnatal age groups (Fig. 5).

In comparison at a particular age group between control and testosterone-treated goats, significant variations ($p<0.05$) were found in tunica albuginea thickness at 2 months, and in seminiferous tubule diameter at 4 months of age of goats, and onward (Fig. 4–5). Seminiferous tubule diameter of control goats at puberty (6m) was approximately 5 times larger than that of day 0 kids during postnatal development, but only slightly more than twice as large (2x) in testosterone-treated goats of the same age group. Moreover, as goats age, their tubular convolution increases and their intertubular (stromal) space decreases; as a result, stromal cells gradually disappear during postnatal development both in control and testosterone-treated Black Bengal goats.

Effects of exogenous testosterone on seminiferous epithelium of postnatal developing testis of Black Bengal goat

The parenchyma of testis from birth or day 0 up to 1 month aged kids was consists of seminiferous tubules scattered throughout the stroma, and the majority of the tubules were spherical, with only a few of the

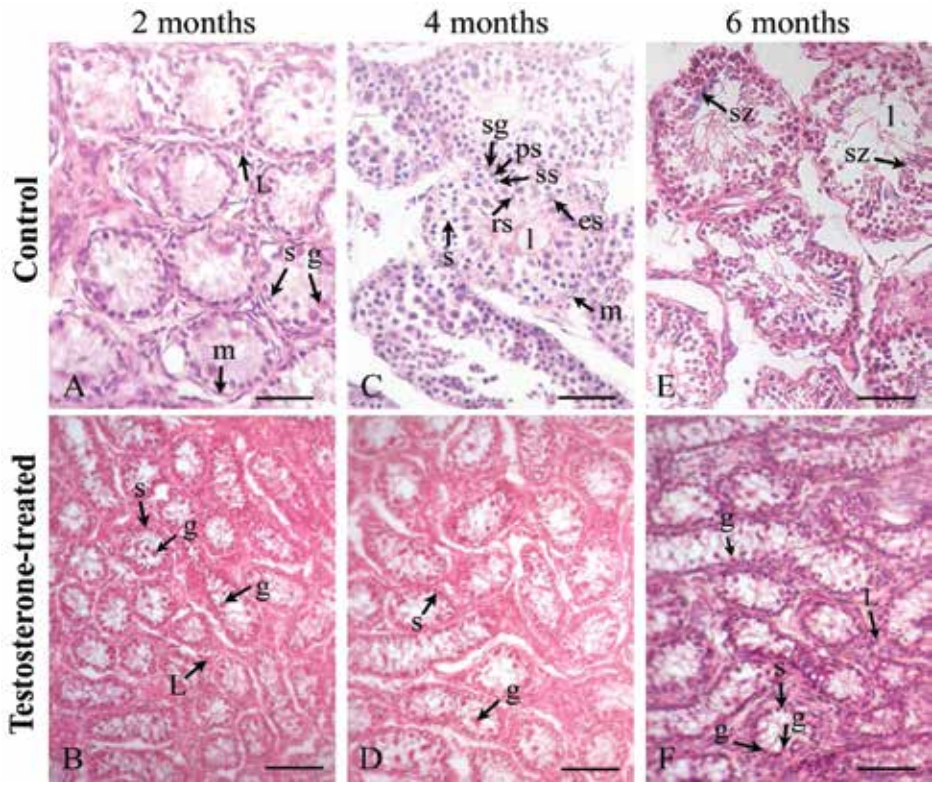

Fig. 6A-F. Micrographs showing the pattern of histological development of seminiferous epithelium in control and testosterone-treated Black Bengal goats during the postnatal developmental stages. Hematoxylin and eosin stains were used to stain the sections. Only Sertoli cells (located in the periphery) and gonocytes (located in the center or close to periphery) with no lumen were present in the seminiferous tubules of two months (2m) aged testis of both groups. The tubules of the four (4m) and six months (6m) old have a distict lumen in control goats. In control goats, at 4m of postnatal age, seminiferous epithelium contained the spermatogonia, primary and secondary spermatocytes, round spermatids, and elongated spermatids adhered with Sertoli cells at their ad luminal border; and at 6m of postnatal age, seminiferous epithelium contained all type of cells of spermatogenic lineage including spermatozoa indicating control Black Bengal goat reaches puberty at this age. In testosterone-treated goats, no stratification of seminiferous epithelium was observed until postnatal 6m of age. Most of the gonocytes (prespermatogonia) took place among the Sertoli cells by 6m of postnatal age. s, Sertoli cell; g, gonocyte; sg, spermatogonia; ps, primary spermatocytes; ss, secondary spermatocytes; rs, round spermatids; es, elongated spermatids; sz, spermatozoa; m, myoid cell; L, stromal cells including Leydig cells; l, lumen; scale bar 50 µm.

convoluted variety in control goats. There was no lumen within the tubules; hence, these tubules were called the sex cords. The interior of the sex cords was filled with acidophilic ground materials. These solid sex cords featured central large germ cells (gonocytes) with spherical centrally positioned nucleus and acidophilic cytoplasm, as well as peripheral Sertoli cells (lying on the basement membrane) with generally ovoid-shaped nucleus and light cytoplasm. Each sex cord had a distinct basement membrane, which was encircled by one or more layers of peritubular myoid cells. In the stroma, the areas between the sex cords, there were numerous interstitial or stromal cells, including Leydig cells.

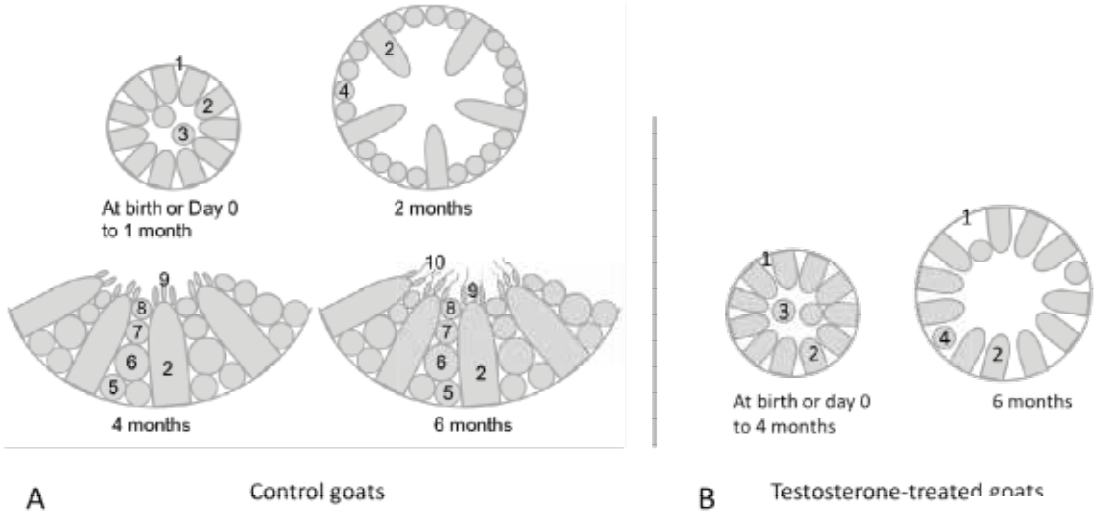

Fig. 7A-B. Schematic diagrams displaying the general postnatal development of seminiferous tubules in the testis of control and testosterone-treated Black Bengal goats. 1, basement membrane; 2, Sertoli cell; 3, gonocyte; 4, prespermatogonia; 5, spermatogonia; 6, primary spermatocytes; 7, secondary spermatocytes; 8, round spermatids; 9, elongated spermatids; 10, spermatozoa.

With the progression of age of goat kids, gonocytes began to migrate toward the basement membrane of the tubules, which later became spermatogonia situated among the Sertoli cells at the periphery of tubules and conforming the basal cell layer of seminiferous tubules by two months postnatal age (Fig. 6A). After 4 months of postnatal age, the seminiferous tubules were shown to have a lumen in the middle (Fig. 6C, E). About 4 months of age, seminiferous tubules containing stratified epithelium that includes the spermatogonia, primary and secondary spermatocytes, round spermatids, and elongated spermatids adhered with Sertoli cells at their ad luminal border were seen (Fig. 6C). About 6 months after birth, seminiferous tubules contained all types of cells of spermatogenic lineage including spermatozoa adhered with Sertoli cells at their ad luminal border and in the lumen (Fig. 6E). The lumen of a few seminiferous tubules from testes that were 4 and 6 months old had a small number of disconnected degenerated/apoptotic cells. The lumen of seminiferous tubules containing spermatozoa, which was revealed by histological studies, suggested that Black Bengal goats reached puberty at 6 months after birth. The general postnatal developmental pattern of seminiferous tubules in the testis of control Black Bengal goats is shown in Fig. 7A.

In testosterone-treated goats, the parenchyma of testis from birth to 2 months old kids was more or less similar with early age in control goats (day 0 to 1 month), which contained peripheral Sertoli cells (resting n the basement membrane) with mostly ovoid-shaped nucleus and light cytoplasm, and central large germ cells (gonocytes) with spherical centrally placed nucleus and acidophilic cytoplasm (Fig. 6B).

With progression of age, the gonocytes began moving in the direction of the seminiferous tubules' basement membrane but the migration was seemed very slow. Most of the gonocytes (prespermatogonia)

were moved centrifugally and took place among the Sertoli cells by postnatal 6 months of age (Fig. 6F). In goats treated with testosterone, stratification of the seminiferous epithelium and lumenization of the seminiferous tubules were not seen until 6 months of age (Fig. 6D, F) indicating that exogenous testosterone hampers the normal postnatal testicular development, and disrupts the process of spermatogenesis. Figure 7B depicts the general postnatal development of seminiferous tubules in testosterone-treated Black Bengal goats up to 6 months after birth.

Spermatogenesis is controlled by the pulsatile release of hypothalamic gonadotropin-releasing hormone (GnRH), which stimulates the anterior pituitary to release follicle stimulating hormone (FSH) and luteinizing hormone (LH) on an episodic basis. Testosterone is produced when LH stimulates the Leydig cells to do so. This testosterone then has a local impact on the testicular stroma and seminiferous tubules, including Sertoli cells, and leads to the production and maturation of sperm. When compared to bloodstream levels, intratesticular testosterone (ITT) is extremely high, indicating this effect. The FSH promotes spermatogenesis by acting directly on the Sertoli cells (Basaria, 2014).

The results of our postnatal developmental study demonstrated that in control goats, the testis of Black Bengal goats were developed smoothly and gradually that was evident both in biometrical and histomorphometrical parameters. A substantial increase in testicular length, width, and weight along with a stratification of the seminiferous epithelium and a dramatic increase in the diameter of the seminiferous tubules were noted at 4 months after birth, indicating the initiation of spermatogenesis in control goats. The development of seminiferous epithelium was completed, *i.e*., the completion of the first wave of spermatogenesis was completed by 6 months of postnatal age that was evident as all types of cells of spermatogenic lineage were present including the spermatozoa, which were found attached to the Sertoli cells at their ad luminal border as well as in the lumen. Postnatal study on indigenous sheep testis showed the same smooth and gradual testicular development (Sadi and Royhan, 2022).

Androgens, especially testosterone (converted by the enzyme aromatase in the testicular stroma) has direct negative feedback modulators of GnRH, LH and FSH (Hayes *et al*., 2001). A higher quantity of testosterone causes the hypothalamic-pituitary axis to respond negatively, limiting GnRH release, which then prevents the generation of FSH and LH (Basaria, 2014). Intratesticular testosterone levels and total testosterone production, therefore, decline (Bhasin *et al*., 2018; Crosnoe *et al*., 2013). When synthetic testosterone is administered exogenously, the gonadotropins (FSH and LH) are not released at amounts sufficient for spermatogenesis. High intra-testicular testosterone levels work in conjunction with the effect of FSH on Sertoli cells to promote spermatogenesis. Exogenous testosterone inhibits LH secretion, which in turn inhibits the Leydig cells from producing endogenous testosterone. In the end, the low intra-testicular testosterone level combined with the suppression of FSH results in decreased spermatogonia proliferation, defects in spermiation of mature spermatozoa by Sertoli cells and accelerated apoptosis of spermatozoa (Smith and Walker, 2014; Patel *et al*., 2019). This is evident in our testosterone-treated study in goats.

Exogenous testosterone also causes developmental changes during early phases of prostate organogenesis in gerbils (Ramos *et al*., 2020). Exogenous administration of testosterone in the present study delayed the normal testicular postnatal development in Black Bengal goats. The values of biometrical and histomorphometrical parameters were significantly lower in testosterone-treated goats than that of control goats from 2 months after birth and onward. Exogenous administration of testosterone resulted in low level of intra-testicular testosterone, which inhibited or delayed the proliferation of spermatogonia. Stratification of seminiferous epithelium, *i.e*., the proliferation of spermatogonia as well as lumenization of the seminiferous tubules was not observed until 6 months of age in testosterone-treated goats indicating that exogenous testosterone hampers the normal postnatal testicular development, and disrupts the process of spermatogenesis. Almost similar tubular atrophy of seminiferous tubules and impairment of spermatogenesis in exogenous testosterone-treated rats were found by Abaas (2020). As seminiferous tubules make up about 80% of the testicular volume in pubertal animals, tubular atrophy of these tubules led to testicular atrophy (Amory and Bremner, 2001). We observed grossly testicular atrophy, and histologically seminiferous tubular atrophy which also led to increased thickness of tunica albuginea in testosterone-treated goats.

Conclusions

The testicular development was slow up to 2 weeks of age, and thereafter, a quick growth of the testis was noticed in control goats. In contrast, a slow trend of testicular development was continuous throughout the duration of the study (up to postnatal 6 months) in testosterone-treated goats. In control goats, a continuous and gradual developmental process of testis was observed, and completion of the first wave of spermatogenesis, *i.e*. establishment of spermatogenesis was observed by 6 months of age. Testosterone is indispensible for sustaining male fertility and spermatogenesis, but continuous administration of exogenous testosterone exerted a negative effect on the postnatal developmental process of testis of goats. The postnatal developmental process of testis was very slow. The stratification of seminiferous epithelium, *i.e*. the proliferation of spermatogonia as well as lumenization of the seminiferous tubules was not observed until 6 months of age in testosterone-treated goats indicating that exogenous testosterone delay the normal postnatal testicular development, and disrupts the process of spermatogenesis.

Conflict of interest

The authors claim that they have no interests that conflict with one another.

Acknowledgments

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