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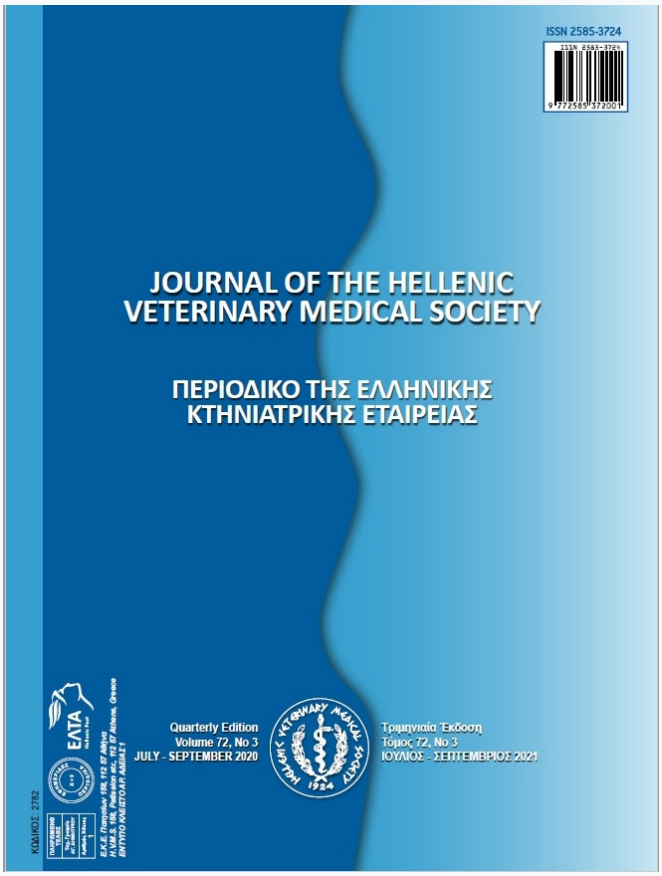

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Y BELKHIRI, S BENBIA, A DJAOUT

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Age related changes in testicular histomorphometry and spermatogenic activity of bulls

Y. Belkhiri[1.2*], S. Benbia[1.3], A. Djaout[2.4]

[1]*Biology of Organisms Department, Faculty of Natural and Life Sciences, University of Batna 2, Algeria*

[2] *Laboratory of Animal Productions, Biotechnologies and Health. Institute Agronomic and Veterinary Sciences, Souk-Ahras University, Algeria*

[3] *Biotechnology's Laboratory of the Bioactive Molecules and the Cellular Physiopathology, University of Batna 2, Algeria*

[4] *Algeria's National Institute for Agricultural Research (INRAA), Algeria*

ABSTRACT: The aim of the present study was to evaluate age related changes in testicular histomorphometry and spermatogenic activity of bulls during their sexual development. A total of 36 bulls were selected and divided into four groups (n=9 in each) according to their age. Bulls included in Groups I, II, III and IV were 10, 12, 14 and 16 months old respectively. Left testes of bulls were subjected to histomorphometry after slaughter. Statistical analysis revealed that the secondary spermatocytes, round and elongated spermatids increased significantly ($P<0.05$) with the age of bulls. Likewise, both sertoli and leydig cell numbers increased significantly ($P<0.05$) with the age of bulls. However, the number of spermatogonia and primary spermatocytes did not change ($P>0.05$) due to age. The mean tubular diameter increased from 200.70±5.45 µm (10 months of age) to 227.30±9.16 µm (16 months of age) and the total volume of seminiferous tubule per testis from 69.63±1.50 % (10 months of age) to 84.64±2.53 % (16 months of age). A positive linear relationship ($P<0.05$) was found between meiotic index (Y) and the age (X, in month), which was characterized by the equation 0.048X+3.135 and a coefficient of correlation (R) of 0.396. The correlation between age and sertoli cell efficiency was 0.482 with a regression equation Y= 0.141X+7.696. It is concluded that histomorphometric parameters of the bulls' testes and spermatogenic activity are correlated with the age, so these parameters provide a reliable tool for the assessment of the reproductive state and sperm production capacity of a bull in a breeding program.

***Keywords*:** histomorphology; spermatogenic efficiency, testis; age; bulls.

Corresponding Author:
Y. Belkhir
E-mail address: y.belkhiri@univ-batna2.dz

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INTRODUCTION

Fertility in bulls is a complex trait that is made up of several physiological processes such as the development of the reproductive system from birth to puberty, spermatogenesis, ejaculation and mating behaviour (which involves libido and copulation). For optimal semen quality, all these physiological processes should be coordinated. Since the bull has more genetic influence (80- 90%) on calves it sires, fertile bull selection can be the most powerful method for improvement of the herd (Rezende et al., 2018)

Anatomical studies on the male genital system at various ages, particularly the testis and its tubular system, are important to understand the anatomical growth, development and physiology of the bulls. To select a breeding bull the anatomy of the testes of young animal and their gradual development is very important. If any anatomical defect is observed at young age, the animal shouldn't be reared as a breeding bull in spite of good genetic characters. Selection for precocious sexual maturation in bulls can decrease production costs, reduce generation interval, and increase genetic gains and overall productivity (Britao et al., 2004)

The relationship between age and reproductive efficiency in bulls has already been evaluated based on testicular biometry parameters, sexual behavior and sperm production and quality (Pant et al., 2003; Luz et al., 2013; Ahmad et al., 2010; Genedy et al., 2019). It is known that the quantification of spermatogenesis, which consists of the numerical observation of different types of cells and constituents of the seminiferous epithelium and ratios of different cell populations, is an important method of sperm production analysis (Berndtson and Picket, 1987). However, quantitative data on spermatogenesis efficiency, measurements of the tubular diameter, seminiferous epithelium thickness, during sexual development are still inadequately documented. The quantitative histology of the testicles, efficiency of sertoli cells, and general morphometry of the seminiferous tubule may be related to the quantity of spermatozoa in the ejaculate, as well as reproductive efficiency (Kalwar et al., 2020). Information on the process of spermatogenesis should provide a basis for experimental work to promote increased spermatogenic activity in the testes and to achieve puberty at an earlier age. The aim of the present study was to quantify the changes in the seminiferous tubules during sexual development and relate them with age of bulls. The data of testicular activity can be used for the definite selection of bulls in future breeding programs.

MATERIALS AND METHODS

Location

The experiment was carried out in Batna province in Algeria, which is located between 35°33′21″ North latitude and 6°10′26″ East longitude and at an altitude range from 968 m mean sea level. Climate in Batna is semi-arid, with four distinct seasons, mean temperature varies between 4C° (January) to 35°C (July) and mean annual rainfall is 329 mm.

General procedures

A total of 36 local breed bulls (non-descript) were selected and divided into four groups according to their age and each group consisted of 9 bulls i.e., Group I: 10 months, Group II: 12 months, Group III: 14 months, and Group IV: 16 months.

Testicular histology

Within 20 minutes after slaughter, the scrotum was incised, the paired testicular weight was estimated by digital scale. Small pieces were taken from three regions (proximal, middle and distal) of the left testes. Immediately fixed in fixed in formaldehyde 10% for 24 hour, and dehydrated subsequently by submerging in series of concentrated ethanol (70%, 95% and 100%). Afterward dehydrated tissues were cleared and infiltrated by placing in liquid paraffin for embedding procedure. Five alternate sections of 5µm thickness from each slide were cut and stained with hematoxylin and eosin. From each section, at least ten essentially circular tubular cross sections for each bull were assessed to determine tubular diameter and epithelial height using AxioVision Rel 4.6 (Carl Zeiss, Thornwood, NY).

The testicular weight (g) for both testes was directly converted into volume (VT), since the volume density of the testes in mammals is very close to one as reported previously by Lunstra et al. (2003). Relative volume (Vr) of seminiferous tubules (surface occupied by the tubules divided by total surface of the field), was determined using the AxioVision software (Carl Zeiss, Thornwood, NY). Total seminiferous tubules volume per testis (VTS) (%) was measured by multiplying Vr by VT. The volume of the intertubular tissue (VIT): was estimated by subtracting the volume of seminiferous tubules from the total testicular volume.

Seminiferous tubule diameter (STD) was measured at X 200 magnification, using a digital camera (MICROCAM MA88-500) attached to the ocular of a light microscope (ZEISS, Germany, Axioplan) and connected to a computer. The epithelium height (GEH) was obtained in the same tubules used for tubular diameter measurements.

Concerning the assessment of total length of seminiferous tubules (LST); the seminiferous tubules were assumed to form a single cylinder with a radius r and a volume VTS; using the equation $VTS=\pi r^2$ LST (Sarma and Devi, 2017).

The different types of germ cells nuclei and sertoli cell nucleoli per tubular cross-section were evaluated at 600 X magnification using light microscopy on10 roundish randomly selected tubular cross-sections per testis. These counts were corrected for section thickness and nucleus or nucleolus diameter according to a previous report (França and Godinho, 2003). For this purpose, 10 nuclei or nucleoli diameters were measured for each cell type analysed per animal. Numerical correction (Nc) of spermatogonia, primary and secondary spermatocytes, round and elongated spermatids, and leydig cells, was performed by using the equation: Nc=COxE/(E+D) where CO is the number of cell nuclei per unit area, E is the average thickness of the section (5 μm), and D is the average nuclear diameter (measured via AxioVision software). Total number of spermatogonia, primary and secondary spermatocytes, round and elongated spermatids, sertoli cells, and leydig cells per testis were determined by multiplying Nc (for each cell type) by VT. This procedure is more precise for the different types of germ cells and round spermatids because they have nuclei with spherical shape. On the other hand, it is less precise for elongated spermatids and sertoli cells of bulls because these cell nuclei have shapes that are not quite spherical (Hien et al., 2011).

The efficiency of the spermatogenesis process was evaluated through several quantitative parameters based on the counting of various cells of the seminiferous tubules, as reported by Segatelli et al. (2004) and Pintus et al. (2015):

Ratio	Functional aspects
i) Primary spermatocytes / spermatogonia	To estimate the coefficient of efficiency of spermatogonial mitosis
ii) Round spermatid/spermatogonia	To obtain an overall spermatogenesis yield
iii) Round spermatids /Primary spermatocytes	To obtain rate of germ cell apoptosis (loss) during meiosis (Meiotic Index)
iv) Elongated spermatids/ Round spermatids	To estimate the post-meiotic germ loss
v) Round spermatids/ sertoli cells	To estimate sertoli cell efficiency

Statistical analysis

All statistical analyses were performed using SPSS 20.0 (SPSS Inc, Chicago, IL, USA). Observations were grouped according to the age of animals, and means and standard error (SE) were determined. The Kolmogorov-Smirnov and Levene's tests were used to check data normality and homogeneity of variance, respectively. Means were analysed by one way analysis of variance between the different age groups of bulls, followed by Tukey's post hoc test to determine significant differences between the groups. One-tailed Pearson correlation test and regression analysis were used to assess the relationship between various observed parameters and age. A value of $P\leq0.05$ was considered statistically significant. Furthermore, according to the level of statistical significance, results of the present study are presented as follows: $P<0.05$: (*), $P<0.01$: (**) and $P<0.001$: (***), respectively.

RESULTS

Age related changes in histomorphometric testicular parameters

The results of different germ cells number at different age of bulls are presented in Table 1. These results revealed that spermatogonia, primary and secondary spermatocytes, round and elongated spermatid, sertoli cells and leydig cells were present in bulls of all ages (10, 12, 14 and 16 months).

According to age, the sertoli cell number, second-

ary spermatocytes and round and elongated spermatids increased significantly (P<0.05): the maximum numbers of these cells per testis were found in the 16 years age group, followed by the 14, 12, and 10 month age groups. Similarly, mean values for leydig cells number was lower (P<0.01) in the groups I (10 months) compared to group IV (16 months) bulls. However, there was no difference in the mean values of the spermatogonia and primary spermatocytes number between the experimental groups (P>0.05).

The relationship between age (X) and the different germ cells number (Y) is shown in Table 2. The sertoli cell number showed a linear relationship with the age of bulls and was characterized by the equation Y=0.164X+1.848, R=0.543 (P<0.01).

A positive linear relationship (P<0.01) was found between leydig cells number and the age of bulls (Table 2).

Table 1. Number (Mean ± SE) of different cells per testis and per gram of testes of male bulls at different ages.

Age	Paramaters						
	Sertoli cells	Spermatogonia	Primary spermatocytes	Secondary spermatocytes	Round spermatids	Elongated spermatids	Leydig cells
Total number per testis ($x10^9$)							
GI	2.80±0.39*	2.72±0.39 ^{NS}	7.73±0.65 ^{NS}	6.39±0.52*	20.57±1.42*	10.90±4.5*	3.72±0.11**
GII	3.23±0.23*	3.11±0.23^{NS}	8.28±1.10 ^{NS}	7.54±0.88*	21.76±2.4*	17.93±2.1*	3.94±0.19**
GIII	3.92±0.24*	3.81±0.24 ^{NS}	9.55±0.69 ^{NS}	9.07±0.55*	23.09±1.5*	20.17±3.4*	4.93±0.12**
GIV	4.14±0.39*	3.98±0.39 ^{NS}	8.24±1.10 ^{NS}	9.54±0.84*	28.46±2.6*	26.96±4.5*	5.29±0.20**
Total	3.60±0.14	3.47±0.14	8.32±0.39	7.97±0.35	21.62±8.43	23.04±2.11	4.38±0.12
Total number per gram of testis ($x10^6$)							
GI	4.93±0.68 *	4.61±0.42 ^{NS}	10.36±1.41^{NS}	10.48±0.80*	13.98±7.94**	27.72±2.51*	6.126±0.42*
GII	4.77±0.43 *	4.69±0.67 ^{NS}	11.41±0.88^{NS}	12.76±1.34*	28.88±4.72**	27.09±4.04*	6.212±0.42*
GIII	5.42±0.41*	5.22±0.40^{NS}	12.42±0.84^{NS}	11.01±0.84*	32.49±4.95**	33.38±2.40*	6.748±0.26*
GIV	5.56±0.68*	5.37±0.67 ^{NS}	14.16±1.41^{NS}	12.57±1.34*	62.84±7.94**	41.49±4.04*	7.037±0.25*
Total	5.26±0.23	5.06±0.23	11.97±0.49	11.58±0.48	31.71±1.49	34.11±3.40	6.43±0.23

^{NS}: Not significant; *: Significant at P <0.05; **: Significant at P <0.01; ***: Significant at P <0.001.

Table 2. Relationship between the different cells in testis and age of bulls.

Dependent variable (Y)	Regression equation (X=Age)	R
Sertoli cells	Y=0.164X+1.848	0.543**
Spermatogonia	Y= 0.159X+1.777	0.225^{NS}
Primary spermatocytes	Y=0.295X+5.226	0.213^{NS}
Secondary spermatocytes	Y=0.552X+2.247	0.539**
Round spermatids	Y=2.775X+15.447	0.621***
Elongated spermatids	Y=0.291X+19.971	0.465**
Leydig cells	Y=0.163X+2.698	0.438**

^{NS}: Not significant; **: Significant at P <0.01. ***; Significant at P <0.001.

Age related changes in morphometric testicular parameters

Data corresponding to values obtained for total seminiferous tubule volume, total length and diameter of the seminiferous tubules, interstitium volume and seminiferous epithelial height during testicular development from 10 to 16 months of age are presented in Table 3.

The volume percent occupied by the seminiferous tubule differed significantly between the four groups (P<0.05). The seminiferous tubules volume ranged from 69.63±1.50 to 84.64±2.53 %.

The diameters of seminiferous tubule were significantly larger (p < 0.05) in the testes of bulls at 16 months compared to those at 10 and 14 months. The seminiferous tubule diameters at age of 10, 12, 14, and 16 months were 200.7±5.45, 202.0±9.16, 213.2±5.71, and 227.3±9.16μm, respectively. Although the length of seminiferous tubules increased with age, no significant differences was observed between the age groups (P>0.05).

The interstitium volume decreased significantly (P<0.01) with increase in the age of bull whereas the height of germinal epithelium in testes increased with the increase in the age of bulls (P<0.05; Table 3).

Table 3. Morphometrical values (Mean ± SE) of seminiferous tubules of testes in male bulls at different ages.

Age	Parameters				
	Total seminiferous tubule volume per testis (%)	Seminiferous tubules diameter (μm)	Length of seminiferous tubules (m)	Interstitium volume (%)	Germinal epithelium height of testes (μm)
GI	69.63±1.50*	200.70±5.45*	3043.76±201.05^{NS}	20.57±0.74**	79.43±0.75*
GII	70.46±2.53*	201.96±9.16*	3436.45±337.84NS	15.88±1.27**	83.40±1.27*
GIII	84.64±1.57*	213.23±5.71*	3234.25±210.68NS	13.55±0.79**	84.11±1.27*
GIV	84.64±2.53*	227.30±9.16*	3526.43±337.84NS	16.59±1.27**	86.44±0.79*
Total	83.01±0.65	209.01±3.45	3251.71±115.59	16.98±0.65	76.88±1.59

^{NS}: Not significant; *: Significant at P <0.05; **: Significant at P <0.01.

Table 4. Relationship between the morphometric data of testes and age of bulls.

Dependent variable (Y)	Regression equation (X= Age)	R
Total seminiferous tubule volume per testis (%)	Y= 1.158X+71.046	0.626***
Seminiferous tubuli diameter (μm)	Y= 1.741X+191.010	0.520**
Length of seminiferous tubules (m)	Y= 65.013X+2579.391	0.196^{NS}
Interstitium volume (%)	Y= -1.158X+28.954	-0.616***
Germinal epithelium height of testes (μm)	Y= 1.984X+56.342	0.499**

^{NS}: Not significant; *: Significant at P <0.05; **: Significant at P <0.01; ***: Significant at P <0.001.

Relationship of age with morphometric testicular parameters of bulls is presented in Table 4. A positive relationship (P<0.001) was found between seminiferous tubule volume and the age of bulls. This relationship was linear and was characterized by the equation Y= 1.158X+71.046, where Y equals total seminiferous tubule volume in percent, and X denotes the age in month. The coefficient of correlation (R) between these parameters was 0.626 (P<0.001).

Age related changes in the efficiency of the spermatogenesis

Data corresponding to values obtained for the efficiency of the spermatogenesis are presented in Table 5. The efficiency coefficient showed uniformity in the average values found, ranging from 2.34 in GI to 2.55 in G IV (P>0.05). The maintenance of the efficiency of spermatogonial mitosis among the different experimental groups may be confirmed further through the results of the corrected number of cells in a testis on per gram basis as listed in Table 1.

The overall spermatogenic yields was significantly higher (p < 0.01) in the testes of 16 months old bull compared to those of 10 and 14 months old bulls. Again, the Meiotic Index (ratio between round spermatids and pachytene primary spermatocytes) recorded in the present study in bulls ranged from 2.3 ± 0.20 at 10 months to 3.0 ± 0.20 in 16 month-old bulls. The overall Meiotic Index in bulls was recorded as 2.6 ± 0.07. The ratio of elongated spermatids to round spermatids differed significantly (P<0.01) between groups and the measurements increased significantly by the advancement of bull's age. The efficiency of sertoli cells (ratio of round spermatid and sertoli cell nuclei) showed a significant (P < 0.01) increase between 10 and 16 months of age (Groups-I & IV). Relationship of age to efficiency of the spermatogenesis in bulls is presented in Table 6.

Table 5. Efficiency of the spermatogenesis (Mean ± SE) in bulls of different ages.

Age	Parameters				
	Efficiency Coefficient	Meiotic index	Overall Spermatogenesis Yield	Elongated spermatids /Round spermatids	Sertoli cell efficiency
GI	2.34±0.30^{NS}	2.29±0.20*	5.40±0.80**	0.52±0.25**	5.21±0.75**
GII	2.44±0.18^{NS}	2.66±0.11*	6.45±0.43**	0.82±0.25**	5.89±0.45**
GIII	2.43±0.16^{NS}	2.97±0.12*	6.10±0.47**	0.87±0.15**	6.17±0.40**
GIV	2.45±0.30^{NS}	3.04±0.20*	8.88±0.80**	0.94±0.14**	8.46±0.75**
Total	2.48±0.10	2.63±0.07	6.50±0.30	0.78±0.10	6.23±0.28

^{NS}: Not significant; *: Significant at P <0.05; **: Significant at P <0.01; ***: Significant at P <0.001.

Table 6. Relationship between the efficiency of spermatogenesis and age of bulls.

Dependent variable (Y)	Regression equation (X= Age)	R
Efficiency Coefficient	Y= 0.012X+2.611	0.039^{NS}
Meiotic index	Y= 0.048X+3.135	0.396*
Overall Spermatogenesis Yield	Y= 0.157X+8.125	0.378*
Elongated spermatids /Round spermatids	Y= 0.109 X+2.261	0.473**
Sertoli cell efficiency	Y= 0.141X+7.696	0.482**

^{NS}: Not significant; *: Significant at P <0.05; **: Significant at P <0.01.

DISCUSSION

The present study estimates the spermatogenic efficiency and histomorphometry of bull's testis structure during the sexual development in a local non-descript cattle breed. These standards can be used for selection of bulls maintained for breeding programs to eliminate the problem of infertility and could be help in minimizing the age at puberty.

In the present study, the total number of secondary spermatocytes and round and elongated spermatids increased with age of the male (Table 1). Our findings were consistent with those of Karmore et al. (2001), who reported that the number of these cells was lower in prepubertal than pubertal and post-pubertal animals. Sun et al. (2017) reported that cattle yak had similar histomorphological structures at 10, 12, and 14 months of age. In pigs, the number of germ cell per seminiferous cord/tubular cross section was very low from birth to 4 months of age. A very dramatic increase in various populations of germ cells per cross section of seminiferous tubule occurred from 4 to 5 months of age, but the number of various germ cells showed a tendency to stabilize after 7 months of age (França et al., 2000).

In this study, the relationship between bull's age and Sertoli cell number was highly significant. In horses, it has been reported that the Sertoli cells have a stable population size in adults because evidence of Sertoli cell division and Sertoli cell death is not obvious in adults. However, the age-related loss of Sertoli cells in humans was not accompanied by obvious degeneration of Sertoli cells, and mitotic activity at the base of seminiferous epithelium is generally considered spermatogonial in nature (Johnson et al., 1994). Based on the fact that each Sertoli cell supports a limited number of germ cells in a species-specific manner (Almeida et al., 2006), and the number of Sertoli cell is established before puberty; hence, it determines the rate of sperm production in sexually mature animals (Johnson et al., 2008).

Most authors report a decrease in the number of Leydig cells with age (Gofur et al., 2008; Petersen et al., 2015). This stands in contrast with our finding for total number of Leydig cells, which did not decline with age. Karmore et al. (2001) reported that the number of Leydig cells was less in prepubertal than in pubertal and post-pubertal goats. In most mammals, other than seasonal breeders, the Leydig cells undergo a phase of proliferation from resident stem cells during pre-puberty, followed by differentiation, which results in a fixed number of Leydig cells persisting with only modest attrition throughout life (Teerds and Huhtaniemi, 2015). Because the body and testicular weights increased substantially during development (Lee et al., 2004), the demands for steroids and other substances secreted by Leydig cells were probably higher, requiring more Leydig cells per testis.

In the current study, the total seminiferous tubule volume showed changes during testicular development. This might be attributed to cellular proliferation, secretion of tubular fluid, and appearance of the tubular lumen. A similar growth pattern has been reported by Genedy et al. (2019). In general, there is 70 to 90% of the seminiferous tubule volume in mammals (França and Russel, 1998) and the observed seminiferous tubules volume (84.64%) is situated around the mean range observed for mammals.

Our findings revealed that the seminiferous tubule diameter increased with advancing age. The significant increase in diameter between 10 and 16 months indicated fast development of the tubules before sexual maturity. Similar findings were documented in Assam goats (Sarma and Devi, 2017) and cattle (Wrobel et al., 1988). Ibrahim et al. (2013) reported that an increase in the process of spermatogenesis led to an increase in the thickness and diameter of seminiferous. Based on these observations, it is implied that the reproductive capacity of bulls in group IV might be

greater than group I, II and III due to larger seminiferous tubules (Gofuret al., 2008).

Ratio estimation between primary spermatocytes and spermatogonia in the present work revealed that the efficiency of spermatogonial mitosis did not vary significantly between the age groups. Sarma and Devi (2017) revealed that the efficiency of spermatogonial mitosis increased noticeably from 4 months of age onward, with the maximum being in 10-month-old goats. In mammals, only 2 or 3 of 10 expected spermatozoa are produced from differentiated type A spermatogonia, and the highest level of germ cell apoptosis occurs during the spermatogonial phase through a density-dependent regulation and during meiosis due to chromosomal damage (Almeida et al., 2006).

Again, the Meiotic Index recorded in the present study in bulls, denoting a decrease in germ cell loss during meiosis with increasing age of the male. However, further works on this aspect is required as no available literature could be traced to compare with the present findings. The meiotic index total was 2.63±0.07. Similar data have been reported for the goat (2.8±0.3; Leal et al., 2004), gerbil (2.8±0.1; Segatelli et al., 2004), cat (2.6±0.2; Neubauer et al., 2004), jaguar (2.66±1.11; Pintus et al., 2015), despite the fact that in these studies only spermatocytes in prophase were considered.

The expected ratio of elongated spermatids to round spermatids is theoretically 1.0 because spermiogenesis does not involve further cellular divisions (Pintus et al., 2015). It was observed that this ratio was only 0.52±0.25 in the experimental Groups I and was significantly lower compared to the Group IV. The lowest ratio of elongated spermatids to round spermatids suggests that a greater selective spermiogenesis during the breeding season may be useful to minimise low quality sperm cells in the ejaculate. However, no such reports were found available in the literature to compare the present findings.

The efficiency of Sertoli cells increased from 10 to 16 months of age in bulls. However, the present mean value for efficiency of Sertoli cell was relatively low when compared with other species such as Assam goats and gerbil (Segatelli et al., 2004; Sarma and Devi, 2017). The noticeable flexibility among species in the number of spermatids supported by a single Sertoli cell shows that, in general, species in which the ratio of spermatids to Sertoli cells is higher may also have a higher spermatogenic efficiency (Sharpe, 1984; França and Russell, 1998).

CONCLUSION

In the present investigation, we obtained several fundamental data regarding the testis structure and several indices that quantify testicular activity in the bull. In conclusion, histomorphometric parameters of the bulls' testes and spermatogenic activity can vary depending on age, but the spermatogonia, primary spermatocytes number and the length of seminiferous tubules were stable during sexual development. It can be recommended that the relationship between the stereological testicular parameters and age may be a reliable tool for the assessment of the reproductive state and sperm production capacity of bull. Thus testicular activity can be used for the definite selection of bulls in future breeding programs.

CONFLICT OF INTEREST STATEMENT

The author declares that there is no conflict of interests regarding the publication of this paper.

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