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Testicular Histomorphometric Evaluation of Zebu Bull Breeds

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ABSTRACT

The objective of this study was to evaluate the quantitative histology and testicular biometrics in zebu bulls of different breeds. Testicular fragments of Nelore (n=10), Polled Nelore (n=6), Gir (n=5), Guzerat (n=5) and Tabapuã bulls (n=5) were used. The fragments were perfusion-fixed in Karnovsky solution, embedded in glycol methacrylate and stained with toluidine blue-1% sodium borate. The Nelore animals had a higher tubular volumetric proportion (85.2%) and greater height of the seminiferous epithelium (73.2 µm) than the Gir, Guzerat and Tabapuã breeds. The Nelore animals also had a higher volumetric proportion of Leydig cells (5.2%) than the Guzerat and Tabapuã breeds. There was no significant difference for any of these parameters between the Nelore and Polled Nelore breeds. The gonadosomatic index, seminiferous tubule diameter, cross-sectional area of the seminiferous tubule and tubule length (total length and length per gram of testicular parenchyma) did not vary among the breeds studied. The morphometric parameters evaluated suggested that the genetic selection applied to the Nelore and Polled Nelore breeds improved the efficiency of spermatogenesis in these breeders.

Key words: cattle, testis, volumetric proportion, seminiferous tubule

INTRODUCTION

Most of the cattle population of the world is kept in tropical and subtropical regions, of which *Bos indicus* and crossbred *Bos indicus* x *Bos taurus* predominate. Zebu animals are better adapted to the tropical climate and are more resistance to thermal stress and endo- and ectoparasites. They are also productive even on a diet of low quality forage (Turner 1980; Hansen 2004). It is estimated that around 80% of the herd of bovine in Brazil is comprised of animals of the zebu breeds and crossbred zebu, showing its great adaptability to environmental conditions and importance in the Brazilian economy (ABCZ 2013).

Despite the rusticity and adaptability of zebu bulls to the Brazilian conditions, it has been reported that zebu bulls were developed later than the European breeds (Vale-Filho et al. 1986). Currently, a major breakthrough in the genetic improvement of zebu animals has occurred, which has decreased the difference between zebu production rates and those of to the European breeds. This effect is observed in the reduced age at puberty and sexual maturity of zebu animals even in those herds that are raised extensively (Brito et al. 2004; Martins et al. 2011; Pinho et al. 2013). This genetic improvement can also be observed in the increased scrotal circumference of

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selected herds (Viu et al. 2006; Martins et al. 2011; Pinho et al. 2013).

The use of the scrotal circumference as the main parameter for the selection of bulls for breeding involves selecting the animals with higher sperm production and an increased testicular volume, which directly implies changes in the functional morphology of this organ and justifies the need for periodic updates of these measurements to characterize the evolution of the morphometric indexes of the testicular parenchyma (Unanian et al. 2000; Andreussi et al. 2013).The volumetric proportion of Leydig cells in the intertubular tissue and the quantitative parameters directly related to the seminiferous tubule, such as the tubule diameter, the thickness of the seminiferous epithelium and the tubule length (total length and length per gram of testicular parenchyma), are positively correlated with spermatogenic activity, providing information regarding the level of spermatogenic activity and constitute indicators of this activity in investigations involving testicular function (França and Russell 1998; Paula et al. 1999).

Despite the great importance of zebu cattle in tropical regions, there are few published studies on the process of spermatogenesis in these animals. The study of Cardoso and Godinho (1983), who described in detail the kinetics and quantification of spermatogenesis in Nelore bulls, can be highlighted.After many years of genetic improvements applied to the Nelore breed, it is possible that some of the results obtained by Cardoso and Godinho (1983) have already been superseded. Furthermore, publications describing the process of spermatogenesis in other breeds of zebu bulls are rare. Despite the lower contribution of these zebu breeds to the total cattle population in Brazil, these breeds participate effectively in this context, especially when crossbred with European breeds.

This study aimed to evaluate some parameters of quantitative histology and testicular biometry, such as the gonadosomatic index, volumetric proportion of the testicular parenchyma components (seminiferous tubules, intertubular tissue, stroma and Leydig cells), tubule diameter and cross-sectional tubular area, height of the seminiferous epithelium and tubule length (total length and length per gram of testicular parenchyma) in five breeds of zebu cattle that are raised in Brazil.

MATERIALS AND METHODS

Animals, testicle collection and assessment of testicular measurements

Testicles were collected from 31 zebu bulls: pure Nelore cattle (n=10), Polled Nelore (n=6), Gir (n=5), Guzerat (n=5) and Tabapuã (n=5), age (mean) and amplitude variation of 6.3 (4–9); 7.4 (6–9); 8.4 (7–10); 7.0 (4–9); and 7.6 (6–9) years, respectively. All the animals were initially subjected to semen evaluation and a full soundness examination to make sure they did not have any changes detectable on clinical examination of the genitals. The selected animals were sexually mature bulls that had semen with at least 50% of sperm exhibiting progressive motility and a sperm morphology with a maximum of 10% with major sperm defects and a maximum of 20% with minor defects (Garcia et al. 1987). The selected animals were orchidectomized and all of the testicles were collected, separated from the epididymis and weighed on a precision scale. One testicle of each animal was frozen and then dissected to determine the percentage occupied by the tunica albuginea and the testicular mediastinum.All the procedures were approved by the Ethical Committee of the Federal University of Mato Grosso do Sul (protocol number 441/2012).

Histological processing

Immediately after dissecting and weighing the epididymis, one of the testicles from each animal was perfused with Karnovsky solution for fixation (Costa and Paula 2006). Testicular fragments of 2.0 x 5.0 x 5.0 mm were collected and embedded in glycol methacrylate according to Costa et al. (2011). Histological sections, 4 μm thick, were made using a glass knife with a rotary microtome. The histological sections were stained with toluidine blue solution–1% sodium borate, and the slides were mounted with Entellan® (Merck, São Paulo, Brazil).

Weight of the testicular parenchyma and gonadosomatic index

After the removal of the epididymis and the dissection of one testicle from each animal, the weight of the testicular parenchyma was calculated by subtracting the weights of the tunica albuginea and the mediastinum from the testicular weight. Because testicular density was very close to 1 g/cm^3 (Johnson et al. 1981), the testicular volume was considered equal to its weight. The

gonadosomatic index represented the percentage of body mass that was allocated to the testicles.

Volumetric proportion of the testicular parenchyma components

The volumetric proportions of the seminiferous tubules, Leydig cells, stroma (comprises connective tissue cells and fibers, nerves, blood and lymph vessels) and intertubular tissue were obtained using the ImageJ 1.34s software (Rasband 2005) with a graticule containing 420 intersections. The dots coincident with Leydig cells, connective tissue cells and fibers, blood vessel lumen, blood vessel wall, lymphatic space, tunica propria, seminiferous epithelium and tubular lumen that were examined in twenty randomly chosen fields by horizontal scanning of the histological sections were considered. Thus, the volumetric proportions, expressed in percentages, were calculated based on a total of 8,400 dots per testicle. A 400x magnification was used in this analysis.

Diameter of the seminiferous tubules, seminiferous epithelium height and cross-sectional area

The mean diameter of the seminiferous tubules was obtained from the cross-sections of tubules that were more circular, regardless of the stage of the seminiferous epithelium cycle (SEC). The measurements were made with the morphometry software ImageJ 1.34s (Rasband 2005). The height of the seminiferous epithelium was measured in the same cross-section in which the diameter of the seminiferous tubules was measured. The distance considered was from the basal membrane to the luminal border; two measurements were made in each cross-section and the mean was recorded. The cross-sectional area of the seminiferous tubule was calculated using the "area calculator" function of the ImageJ 1.34s software (Rasband 2005). Twenty cross-sections of tubules in each animal were assessed.

Length of the seminiferous tubules: total and per gram of testicular parenchyma

The total length of the seminiferous tubules was calculated according to the method of Attal and Courot (1963). Twenty cross-sections of seminiferous tubules were measured, regardless of the stage that the tubules exhibited. The values for the cross-sectional area and the total adjusted volume of the seminiferous tubules were estimated assuming a 5% linear shrinkage factor of the tissue (Amann 1981). The total length of the tubes was expressed in meters. The tubular length per gram of testicular parenchyma was obtained by dividing the total length by the testicular parenchyma weight.

Statistical analysis

The means and standard deviations were calculated with the statistical functions of Microsoft Office Excel. The means were compared using the Tukey test from the statistical program BioEstat 3.0 (Ayres et al. 2003). The level of significance was set to 5% ($p<0.05$).

RESULTS

The age of the animals studied ranged from 4 to 10 years with a mean of 7.2 years and with no difference ($p>0.05$) between the breeds. The Nelore and Polled Nelore breeds had the highest mean body weight, which was not significantly different between these two breeds. However, differences between this maximal mean body weight and those of the other studied breeds were observed. The mean body weight did not vary significantly between the Gir, Guzerat and Tabapuã breeds (Table 1).

Table 1 - Body weight, testicular and testicular parenchyma weights and the gonadosomatic index (GSI) from different Zebu bulls (Mean ± SD).

Breed	Body Weight (kg)	Total Testicular Weight (g)*	Testicular Parenchyma Weight (g)**	GSI (%)
Nelore	575.6 ± 28.48^{a}	372.8 ± 52.92^{a}	342.2 ± 48.57^{a}	0.13 ± 0.01^{a}
Polled Nelore	540.7 ± 45.64^{a}	332.8 ± 24.73^{ab}	305.3 ± 22.69^{ab}	0.12 ± 0.00^{a}
Gir	483.4 ± 11.17^{b}	291.2 ± 35.51^{b}	267.2 ± 32.58^{b}	0.12 ± 0.01^{a}
Guzerat	466.2 ± 14.61^{b}	302.4 ± 25.50^{b}	277.2 ± 23.38^{b}	0.13 ± 0.01^{a}
Tabapuã	466.0 ± 14.73^{b}	298.0 ± 22.23^{b}	273.6 ± 20.41^{b}	0.13 ± 0.01^{a}

* Weight of only one testicle. ** Total testicular weight – Albuginea and mediastinum weight.
Means followed by different letters in the same column differ according to the Tukey test ($p \leq 0.05$).

The mean weights of the testicles and the testicular parenchyma were higher ($p<0.05$) in the Nelore animals (372.8 and 342.2 g, respectively), and the weights did not differ from the mean values obtained in Polled Nelore animals (332.8 and 305.3 g, respectively). No differences were observed ($p>0.05$) between the Polled Nelore, Gir, Guzerat and Tabapuã breeds (Table 1). The tunica albuginea and mediastinum together represented approximately 8.2% of the testicular volume in all of the breeds. The mean gonadosomatic index among the breeds was 0.13 ± 0.01 with no significant differences between them (Table 1).

The volumetric proportions of the components of the testicular parenchyma were not uniform among the evaluated breeds. The tubular volumetric proportion did not vary among the Polled Nelore, Gir, Guzerat and Tabapuã breeds but was higher ($p<0.05$) in the Nelore breed compared to the other breeds, except Polled Nelore. Consequently, the volumetric proportion of intertubular tissue was lower ($p<0.05$) in Nelore cattle, except when compared to Polled Nelore animals (Table 2).

The intertubular compartment contained 4.7 ± 0.5% Leydig cells, and the mean value for the percentage of Leydig cells did not differ ($p>0.05$) between the Nelore, Gir and Polled Nelore animals, nor between the Polled Nelore, Gir, Guzerat and Tabapuã animals. The Nelore breed had a higher ($p<0.05$) volumetric proportion of Leydig cells (5.2%) in the intertubular tissue than the Guzerat (4.3%) and Tabapuã (4.4%) breeds (Table 2). The volumetric proportion of testicular stroma, which included the connective tissue cells and fibers, nerves, blood and lymph vessels, was 11.7 ± 2.2% (intertubular compartment, except Leydig cells). There was no significant difference between the Nelore and Polled Nelore animals or between the Polled Nelore, Gir, Guzerat and Tabapuã animals (Table 2).

The mean diameter of the seminiferous tubules varied slightly in the studied breeds, from 246.6 to 257.9 μm, with no significant differences between the breeds (Table 3).

The seminiferous epithelium had an average height of 70.9 ± 2.2 μm (mean ± standard deviation) among the breeds. Little difference was observed between the breeds; however, the height of the seminiferous epithelium in the Nelore breed was significantly higher than that in the Gir, Guzerat and Tabapuã breeds, but was not different compared to the Polled Nelore breed. There was no difference ($p>0.05$) between the Polled Nelore, Gir, Guzerat and Tabapuã breeds (Table 3).

Table 2 - Volumetric proportion (%) of the components of the testicular parenchyma of different zebu bulls (Mean ± SD).

Breed	Seminiferous Tubule	Leydig Cells	Stroma*	Intertubular Compartment
Nelore	85.2 ± 1.80^{a}	5.2 ± 0.35^{a}	9.6 ± 1.76^{a}	14.8 ± 1.81^{a}
Polled Nelore	84.0 ± 1.91^{ab}	4.8 ± 0.59^{ab}	11.2 ± 1.77^{ab}	16.0 ± 1.92^{ab}
Gir	81.8 ± 1.19^{b}	4.7 ± 0.44^{ab}	13.5 ± 1.35^{b}	18.2 ± 1.19^{b}
Guzerat	82.5 ± 1.07^{b}	4.3 ± 0.18^{b}	13.2 ± 1.06^{b}	17.5 ± 1.07^{b}
Tabapuã	82.3 ± 0.48^{b}	4.4 ± 0.43^{b}	13.3 ± 0.58^{b}	17.7 ± 0.48^{b}

* The stroma comprises connective tissue cells and fibers, nerves, blood and lymph vessels. Means followed by the different letter in the same column differ by the Tukey test ($p<0.05$).

Table 3 - Seminiferous tubule diameter (STD), seminiferous epithelium height (SHE), cross sectional area of the seminiferous epithelium (CSA), tubular length per testicle (TLT) and tubular length per gram of testicular parenchyma (TLGTP) of different zebu bulls (Mean ± SD).

Breed	STD* (μm)	SHE (μm)	CSA* (μm^2)	TLT (m)	TLGTP (m)
Nelore	257.0 ± 7.2^{a}	73.2 ± 1.4^{a}	51914.1 ± 2914.1^{a}	5639.6 ± 891.7^{a}	16.5 ± 0.7^{a}
Polled Nelore	251.5 ± 6.2^{a}	71.0 ± 1.8^{ab}	49688.6 ± 2443.1^{a}	5182.0 ± 509.8^{a}	16.9 ± 0.6^{a}
Gir	246.6 ± 7.2^{a}	69.5 ± 1.6^{b}	47785.0 ± 2796.2^{a}	4564.8 ± 325.2^{a}	17.2 ± 1.0^{a}
Guzerat	247.9 ± 8.3^{a}	69.3 ± 1.6^{b}	48300.5 ± 3225.8^{a}	4782.8 ± 705.4^{a}	17.2 ± 1.2^{a}
Tabapuã	248.6 ± 4.7^{a}	69.0 ± 0.8^{b}	48536.0 ± 1831.0^{a}	4656.0 ± 466.4^{a}	17.0 ± 0.7^{a}

*5% Adopted coefficient of linear shrinkage, according to Amann (1981). Means followed by the different letters in the same column do differ by the Tukey test ($p<0.05$

The mean cross-sectional area of the seminiferous tubules in the studied breeds was 49,689.7 ± 3,035.9 μm^2 (mean ± standard deviation), ranging from 51,914.1 μm^2, in the Nelore to 47,785.0 μm^2 in the Gir breed, and there was no significant difference between all of the evaluated breeds (Table 3). The Nelore breed had the highest testicular weight; tubular length per testicle was also higher in this breed (5,639.6 meters). However, this did not differ ($p>0.05$) from the other breeds. The tubular length per gram of testicular parenchyma ranged from 16.5 to 17.2 meters and was not significantly different between the evaluated breeds (Table 3).

DISCUSSION

In most domestic species, the volumetric proportions of the tunica albuginea and the testicular mediastinum correspond to approximately 10% and 0.5-1.0% of the testicular weight, respectively, (França and Russell 1998), except in carnivores; the volumetric proportion is approximately 18% in domestic cats (França and Godinho 2003) and dogs (Mascarenhas et al. 2006). In the present study, the Nelore animals had the highest mean testicular weight as well as testicular parenchyma weight. Together, the tunica albuginea and the testicular mediastinum occupied approximately 8.2% of the testicular volume in all of the evaluated breeds (8.7% of the volume was tunica albuginea and 1% was mediastinum), which was similar to that reported by Amann (1961; 1962). It is very difficult to isolate and completely remove the testicular mediastinum during the dissection of the testicle; therefore, the values may be underestimated (Costa et al. 2007).

The body weight allocated to the testicles did not differ significantly between the studied zebu breeds. The mean value was 0.13%, which was a rate higher than that observed by Cardoso and Godinho (1983) in Nelore cattle (0.08%) and also that of young animals (17-18 months) of the Shorthorn breed (0.08%, Swierstra 1966). The volumetric proportion of the components of the testicular parenchyma varies considerably among species, directly reflecting the efficiency of sperm production for each breed (França and Russell 1998). In most mammalian species, the seminiferous tubules occupy between 60 and 90% of the testicular volume (Setchell 1982), and the remaining 10 to 40% corresponds to the intertubular tissue, which consists of Leydig cells and stroma (connective tissue cells and fibers, nerves, blood and lymph vessels) (França and Russell 1998).

In the present study a higher tubular volumetric proportion ($p<0.05$) was observed in the Nelore breed compared to the Gir, Guzerat and Tabapuã breeds. The values observed in Nelore (85.2%) and Polled Nelore (84.0%) bulls were higher than those observed by Cardoso and Godinho (1983) in Nelore bulls (81.4%), suggesting that the genetic selection applied to these breeds could have resulted in an increase in the volumetric proportion of the seminiferous tubules in the testicles. Much lower values were observed by Goiozo et al. (2005), who reported 76.4% in pure-origin Nelore breeds, and by Santos et al. (1999), who found 72.0% in zebu bulls without breed information. However, in these studies, the gross testicular weight, without subtracting the weights of the tunica albuginea and mediastinum was considered, which could have resulted in an underestimation of the values. In addition, the animals studied by Santos et al. (1999) apparently were common animals of low genetic merit. Lower values were also observed in Holstein bulls, while a larger tubular volumetric proportion was found in younger animals (77.1-76.5%; 29 months-3 years old) compared to older animals (73.0%, 5.4 to 8.6 years old) (Amann 1962; Almquist and Amann 1962).

The percentage of Leydig cells in relation to the testicular volume is highly variable among animals and can be approximately 1-5% in the rats, mice, chinchillas and guinea pigs and approximately 20-60% in the domestic pig, zebra and mole (Fawcett et al. 1973). Although the Nelore animals had a lower volume of interstitial tissue ($p<0.05$) than the Gir, Guzerat and Tabapuã animals, the volumetric proportion of Leydig cells was significantly higher in the Nelore bulls compared to the Guzerat and Tabapuã bulls. However, the values were close to those found in Nelore cattle (4.8%; Cardoso and Godinho 1983), in various breeds (4.6%; Lennox and Logue 1979), and in young animals (29 months) of the Holstein breed (5.0%; Amann 1962); yet according to other studies, the values were higher than those of Nelore cattle (3.7%; Goiozo et al. 2005) and lower than those observed in Holsteins (7.0%, Amann 1962).

The zebu animals evaluated in this study had a mean diameter of the seminiferous tubules (251.4

μm) similar to that observed in 29-month-old Holstein bulls (254.0 μm) (Amann 1962) and higher than that observed in 4.9-year-old Nelore bulls (232.7 μm; Cardoso and Godinho 1983), 17-18-month-old Shorthorn bulls (210.4 μm; Swierstra 1966) and zebu animals without breed characterization (197.6 μm; Santos et al. 1999). A larger tubular diameter was observed by Amann (1962) in mature Holstein bulls (269.0 μm); however, only two animals were assessed.

The different values observed among the studies could be related to the genetic improvement applied to the breeds, i.e., selecting the animals with increased sperm production. In addition, differences in the methodology, especially regarding the sample used and the correction of calculations due to the linear shrinkage of the tissue that occurs during the processing of the material should also be considered. For example, according to Amann (1981), 15% shrinkage could occur when working with paraffin inclusions. Furthermore, the use of sexually immature animals can interfere with these measurements (Paula et al. 1999).

Under the normal conditions, the mean tubular diameter does not change significantly in sexually mature animals that have no reproductive season (França and Russell 1998). The tubular diameter ranges from 180 to 300 μm for most amniotes (Roosen-Runge 1977).

Information concerning the height of the seminiferous epithelium in cattle is rare. In the present study, the height of the seminiferous epithelium in the Nelore animals was significantly higher than that of the Gir, Guzerat and Tabapuã animals. However, all of the breeds had values close to the small variation observed during the different stages of the cycle of the seminiferous epithelium in cattle (72.0 to 79.0 μm; Wrobel and Schimmel 1989). Lower values (53.4 μm) were reported by Santos et al. (1999) in zebu bulls without breed characterization.

The assessment of the cross-sectional area of the seminiferous tubule has been simplified with the availability of morphometry programs, thus this parameter is commonly assessed in the studies of testicular histomorphometry. Nevertheless, the literature contained no citations of this parameter in cattle. The cross-sectional area of the seminiferous tubule did not differ ($p>0.05$) between the breeds assessed in this study.

The total length of the seminiferous tubules corresponds to the total tubular volume divided by the cross-sectional area of the tubule; thus, it is directly related to the testicle size, and therefore, in this context, the tubular length per gram of testicular parenchyma is a more accurate parameter for comparing spermatogenic capacity. In the animals evaluated in this study, there were no significant differences in the total tubular length or length per gram of testicular parenchyma, and the values were close to those previously observed in Nelore bulls (13.9 meters; Cardoso and Godinho 1983) but higher than those of young Shorthorn animals (10.4 meters; Swierstra 1966). The results obtained in several studies have demonstrated a variation of 10 to 15 meters in domestic mammals (França and Russell 1998).

Comparing the results, there has been some progress on various parameters obtained in Nelore cattle in the present study compared to those of Cardoso and Godinho (1983), such as the gonadosomatic index, volumetric proportion and diameter of the seminiferous tubules and tubular length per gram of testicular parenchyma. In addition to the differences in methodology between the studies, the origin of the animals used by Cardoso and Godinho (1983), common Nelore, might have negatively influenced the results compared to the purebreds of this study. Moreover, during the elapsed three decades, the genetic improvement applied to the zebu breeds in Brazil might have contributed to the increase of the parameters related to spermatogenic capacity, mainly in the Nelore breed.

In all of the comparisons, the animals evaluated by Santos et al. (1999) had lower numbers. Apparently, the bulls in Santos' study were common animals, not subjected to the selection process for improving the reproductive traits, which could suggest that the lower values were related to the degree of genetic improvement of the corresponding animals.

When the results obtained in the present study were analyzed, it was observed that, although numerically higher in the Nelore bulls, there were no statistically significant differences in any of the evaluated parameters between this breed and the Polled Nelore breed. Such similarity between the Polled Nelore and Nelore breeds could be explained by the facts that the first breed arose as a variety of the second, according to the genealogical records beginning in 1969 (Santos

1998), and the low magnitude of the genetic distance between these two breeds (Vozzi et al. 2006). The Polled Nelore breed is currently the second in semen marketing in Brazil among zebu breeds (ASBIA 2011).

Through the content of the bulls' summaries published in Brazil, it could be concluded that the Nelore breed, among other zebu breeds, was the one that underwent higher genetic selection in this country. According to these, in 1984, only 395 Nelore bulls and no Gir, Guzerat or Tabapuã bulls were evaluated. In 2006, 30,820 Nelore bulls were evaluated, while only 3,067 Gir, 3,267 Guzerat and 2,025 Tabapuã bulls were evaluated (Rose et al. 2009). In 2012, 41,912 Nelore bulls were evaluated compared to only 3,557 Gir, 4,314 Guzerat and 2,795 Tabapuã bulls (ABCZ 2013).Furthermore, in Brazil in 2011, 3,017,815 doses of Nelore semen were sold versus 258,868 doses of Polled Nelore, 13,041 doses of Gir, 169,335 of doses of Guzerat and 84,061 of doses of Tabapuã semen (ASBIA 2011). Significantly higher values in most parameters were observed in Nelore bulls compared to Gir, Guzerat and Tabapuã bulls, and by coincidence, these latter breeds were involved with less semen commercialization in Brazil (ASBIA 2011). Theoretically, the selection process applied to each assessed breed could be related to these results.

CONCLUSION

In conclusion, these findings indicate that the parameters of quantitative histology and testicular biometry in Nelore breed were similar to the Polled Nelore breed and better than in Gir, Guzerat and Tabapuã breeds.

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