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Histopathological and histomorphometric testicular characteristics associated to reproductive condition in *Bos indicus* (Nellore) bulls

Características histopatológicas e histomorfométricas testiculares associadas à condição reprodutiva em touros *Bos indicus* (Nelore)

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Abstract

The aim of this study was to evaluate morphological, morphometric and functional aspects of spermatogenesis based on reproductive conditions of Nellore bulls. The study used 25 bulls *Bos indicus* (Nellore), which were classified as satisfactory (n=10) and unsatisfactory (n=15) for reproduction. After orchiectomy, fragments of the right testis of each animal were processed routinely and stained with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS). The slides were analyzed in bright field microscopy at different magnifications. The tubular segments were classified into four levels (normal, mild and moderate degeneration, severe degeneration, and tubular hypoplasia) based on the organization and architecture of the tubular tissue. The average height of the seminiferous epithelium, the average thickness of propria tunic and the average tubular diameter were obtained and the proportion of seminiferous tubules, interstitial tissue, lymphatic vessel and blood vessel of each bull testicle were recorded. Furthermore, the frequency of the three classifications of the spermatogenic cycle A, B and C (A = group of stages I, II, and III which comprise the initial phases of the cycle and mitotic proliferation, B = group of stages IV and V, which comprise intermediate phases and intensive mitosis, C = group stages VI, VII and VIII which comprise the final phases and post-meiosis) was compared. The results showed that the degenerative changes in germ cells are associated with histomorphometric variations, including those in the density of the structures that comprise the testicular parenchyma, and are more representative in bulls with poor semen quality. In addition, the unsatisfactory bulls for reproduction showed reduced meiotic potential compared to that of satisfactory reproductive bulls.

Key words: Bovine, histopathology, testicular morphometry

Resumo

O objetivo neste estudo foi de avaliar aspectos morfológicos, morfométricos e funcionais da espermatogênese com base na condição reprodutiva seminal em touros Nelore. Foram utilizados 25 touros *Bos indicus* (Nelore), os quais foram classificados em aptos (n=10) e inaptos (n=15) à reprodução.

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Após orquiectomia, fragmentos do testículo direito de cada animal foram processados segundo técnicas de rotina e corados com Hematoxilina e Eosina (HE) e Ácido Periódico de Shiff (PAS). As lâminas foram analisadas em microscopia de campo claro em diferentes aumentos. Os segmentos tubulares foram classificados em quatro níveis (normal, degeneração leve e moderada, degeneração acentuada, e hipoplasia tubular) baseados na organização e arquitetura do tecido tubular. Foi obtida a média da altura do epitélio seminífero, da espessura da túnica própria e do diâmetro tubular dos segmentos e foi registrada a proporção de túbulo seminífero, tecido intersticial, vaso linfático e vaso sanguíneo dos testículos de cada touro. Além disso, quantificou-se a frequência das três classificações do ciclo espermatogênico A, B e C (A=agrupamento dos estágios I,II, e III que compreendem as fases iniciais do ciclo e proliferações mitóticas; B= agrupamento dos estágios IV e V que compreendem as fases intermediárias e intensas mitoses; C= agrupamento dos estágios VI, VII e VIII que compreendem as fases finais e de pós-meioses). Os resultados demonstraram que as alterações de caráter degenerativo nas células germinativas estão associadas a variações nos padrões histomorfométricos incluindo a densidade das estruturas que compõem o parênquima testicular e são mais representativas em touros com baixa qualidade seminal. Além disso, os touros inaptos apresentaram redução no potencial meiótico quando comparados aos touros aptos à reprodução.
Palavras-chave: Bovino, histopatologia, morfometria testicular

Introduction

Reproductive efficiency in a herd is directly associated to productivity and profitability in a production system. Thus, fertility is undoubtedly one of the most important characteristics to be considered in dairy and beef programs. As one bull normally mates with many cows, in natural breeding or through artificial insemination, it is evident that male fertility is much more important than that of any individual female (MATO GROSSO DO SUL, 2010).

Several studies have shown a significantly high culling rate of animals with clinical genital alterations and poor semen quality. Studies in Mato Grosso do Sul have shown that the frequency of unsatisfactory bulls, due only to seminal restrictions, evaluated prior to the breeding season, can vary between 15% and 20% in Nellore bulls (NOGUEIRA et al., 2006) and can exceed 35% in animals *Bos indicus x Bos taurus* in other regions of Brazil Brasil (MORAES et al., 1998). Therefore, a significant problem is the permanence of these bulls within a herd as well as the ability to reverse seminal parameters. It is estimated that approximately 43.0% of culled bulls have an advanced degree of degenerative lesions and testicular hypoplasia (FERNANDES et al., 2010).

The decrease in seminal quality standards is directly associated with the conditions of spermatogenesis, which correspond to the endocrine and structural variations of testicular tissue. Furthermore, the production of sperm depends on the coordination between mitotic divisions of spermatogonia and meiotic divisions of spermatocytes, resulting, after a differentiation process, in the spermatids generation. Failures in mitotic or meiotic divisions during spermatogenesis occur frequently in cattle because of the germ cells degeneration, resulting in decreased sperm production and increased percentage of abnormal cells (JOHNSON et al., 2000; HOFLACK et al., 2008).

Histological evaluation is a useful tool to quantify and qualify spermatogenesis in bulls. Quantification can be assessed by morphometric analysis of different measures, the purpose of which is to compare structural changes in cells and tissues, and the density of parenchymal structures (MICKLEM; SANDERSONS, 2001). Alternatively, the systematic qualitative analysis of testicular structures, based on histopathological changes, may provide important information for the study of testicular function bringing new insights to the pathophysiology of

subfertility in bulls. The aim of this study was to evaluate the morphological, morphometric, and functional aspects of spermatogenesis based on the reproductive condition of Nellore bulls.

Material and Methods

Animals and experimental groups

Nellore bulls (n = 25), 3-8 years old, were raised in extensive conditions and used in a natural mating system on a private farm in Campo Grande, MS (20°26′34″S, 54°38′47″W). Clinical signs indicative of infectious diseases were not observed in any of the bulls and these were not previously been in natural mating. After two breeding soundness examinations (30-day interval), the bulls were classified according to the Brazilian Society of Animal Reproduction (CBRA, 1998). Briefly, after clinical examination (general and reproductive tract), semen was collected by electro-ejaculation with penis exposition and rejection of the first fraction. Immediately after collection, the volume (mL), density (creamy, milk-like, skim-like, and translucent), motility (0-100%), and vigor (0-5) of semen samples were evaluated. The samples were preserved in formol-saline 1% for sperm morphology evaluation using a phase-contrast microscopy (×1000, Olympus BX45; Olympus America Inc., Center Valley, PA, USA). Subsequently, bulls were classified according to seminal quality as either satisfactory (minimum 50% motility, vigor 3, 70% morphologically normal sperm; n = 10) or unsatisfactory (inferior parameters; n = 15).

Finally, all bulls were orchiectomized according to Wolf (1986). Middle and cranial testicular fragments (1.0 cm^3) were collected from the right testes and fixed in Bouin's solution for 24 h, washed with running water, and embedded in alcohol 70%. Next, the fragments were histologically processed, embedded in paraffin, and sectioned at 5 µm. Slides were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff slides for evaluation (CARSON; HLADIK, 2009).

Histopathological analysis

Testicular structures were assessed according to the level of tubular and interstitial organization. Under bright field light microcopy (×200 magnification), 100 seminiferous tubule segments of each bull were randomly selected and classified as follows. Normal (NR), segments with normal architecture, symmetric contour, and germ cells well adhered to the seminiferous epithelium; discrete and moderate degeneration (DMD), segments that exhibited discrete or moderate detachment/loosening of germ cells niches, discrete vacuolization, pyknotic nuclei associated with tunica propria, and preserved tubular contour; marked degeneration and necrosis (MD), when tubular segments displayed severe disorder of germinal epithelium, tubular contour, severe vacuolization, loss or necrosis of germ cells, and eventually presence of multinucleated giant cells, associated or not with thickened tunica propria; hypoplastic tubules (HT), with reduced diameter, reduced number or absence of germ cell lineages, Sertoli cells with cytoplasmic processes, and occasionally spermatogonias without mitotic activity.

Histomorphometry and tissue density

The analyses were performed in Haematoxylin and Eosin (H&E) stained slides from images (1024 × 768 pixels) processed in Motic Image Plus 2.0 (Motic Asia, Hong Kong) and ImageJ 1.45M software (ABRÀMOFF et al., 2004). For the morphometry, 30 seminiferous tubule segments were randomly chosen from each bull (×200 magnifications) to determine the average height of seminiferous tubules (µm) and average thickness of tunica propria (µm) in three replicates of each segment. The mean of two transversal measures was used to estimate the tubular diameter (µm). For the density analysis, 15 images randomly chosen from

each bull (×100 magnification) were considered. A grid (266 intersections points) was introduced and the structures present at each intersection, such as seminiferous tubules, interstitial tissue, lymphatic and capillary systems were counted. The relative frequency of each structure was estimated from the image.

Spermatogenic cycle classification

Fifty images (×200 magnification) of seminiferous tubules stained with Periodic Acid-Schiff were used to classify the spermatogenic cycle according to the original stages reported by Cardoso e Godinho (1983): A, clustering of I, II, and III stages composed by initial phases of the spermatogenic cycle enclosing spermatogonias, primary spermatocytes, and some sheaves of elongated spermatids; B, clustering of IV and V stages characterized by secondary spermatocytes (second meiotic division); and C, stages VI, VII, and VIII, relative to the final phases of the spermatogenic cycle, exhibiting two generations of rounded or elongated spermatids and residual bodies in the spermatozoa.

Statistical analysis

Percentages of reproductive histopathological classification attributed to each bull (satisfactory × unsatisfactory) were compared by the Mann-Whitney U test. Linear and histomorphometric measures and tissue density were compared by ANOVA (linear multivariate model) adjusted (covariate) by weight of the bulls. Chi-square (contingence and bipartition tables) tests were used to compare the frequencies of spermatogenic cycle groups between reproductive conditions.

Results and Discussion

The efficiency of spermatogenesis is directly associated with the ability of germ cells to multiply and differentiate. Several physical, chemical, and endocrine factors affect the testicular structure, which can induce mainly the degeneration of the seminiferous epithelium (FOSTER; LADDS, 2007). The decrease in semen quality with the loss of reproductive potential is the main, although widely variable, pathological manifestation of this condition. Bulls with this characteristic are diagnosed as unsatisfactory for reproduction, and represent a significant portion of the economic loss within production systems (BARTH, 2007; NOGUEIRA et al., 2011).

The results clearly show that changes in the organization of the testicular parenchyma affect the tubular density, particularly in the germinal epithelium and during meiotic differentiation. This profile, although with variable intensity, was frequent in the group with poor seminal quality. Transformations in tubular and interstitial structures of the testes are widely found in bulls with testicular degeneration; however, the primary etiology is not always recognized (NASCIMENTO; SANTOS, 1997). Regardless of the cause, classic literature reports the occurrence of these conditions in epidemiological surveys in slaughtered animals or in cases secondary to generalized disease (McENTEE, 1990; FOSTER, 2007; FOSTER; LADDS, 2007).

In this study, we evaluated the progression of the morphological degenerative changes. This pattern ranged from the simple modification of tubular organizational structure to the total loss of germ cells with the possible presence of multinucleated cells. Although descriptive, this analysis enabled classification of the different detected lesions into mild and/or moderate to severe. Additionally, hypoplasia of germ cells was included as a second change, even though it generally appears together with degeneration (KRISHNALIGAM et al., 1982; McENTEE, 1990). These characteristics are associated with different degrees of hypospermatogenesis and have been

used in the systematic analysis of testicular damage in other species (MEISTRICH, 1986; REHM, 2000; MAMINA; ZHIGAL'SKII, 2004; CERELLI et al., 2010).

The frequency of abnormalities and the degree of degeneration was different between the experimental groups. Figure 1 shows that satisfactory bulls had 50.0% to 90.0% normal tubules, whereas only one animal had more than 10% of tubules with severe degeneration and was considered an outlier for that group. However, bulls with poor semen quality, classified as unsatisfactory, had higher percentages of degeneration and tubules with evidence of germ cell hypoplasia. In this group, 43.0% of the seminiferous tubules had at least one change of degenerative character. The frequency of these alterations is associated with the decrease in semen quality, and is possibly irreversible. This percentage is similar to that obtained in a study by Kumi-Diaka et al. (1983), who reported degenerative changes of 40.0% to 69.0% in *Bos indicus* bulls in a tropical environment. In contrast, in satisfactory bulls, the germinal epithelium loss due to degeneration was observed in 28.0% of tubular segments (Figure 1). This value is similar to that reported for Aberdeen Angus bulls [2.9% to 24.6% % (CARROLL; BALL, 1970)] and Belgian Blue bulls [32.0% (HOFLACK et al., 2008)], suggesting that this process is relatively frequent, particularly in bulls raised under extensive conditions (FERNANDES et al., 2010).

Together with the qualitative aspects of the seminiferous epithelium, we evaluated the density of important structures of the testicular parenchyma. This analysis allowed a comparative study between the interstitial tissue and seminiferous tubules, interacting in the remodeling and adaptation to different factors involved in spermatogenesis (MEISTRICH, 1986; CREASY, 1997). However, few studies in bulls have correlated these characteristics with the degeneration of seminiferous epithelium. However, progress of the degenerative process with tubular atrophy and reduction in testicular parenchymal density is frequent (FOSTER; LADDS, 2007).

Bulls with testicular histological changes show hyperplasia of interstitial tissue related to the presence of atrophic areas of Leydig cells and reduction in tubular diameter and volume (RAO VEERAMACHANENI et al., 1986). This fact may explain, in part, the reduction in tubular density. In the present study, although the presence of morphological changes in Leydig cells was not evaluated, the results showed that there was a significant reduction in the density of seminiferous tubules, in contrast to the higher percentage of interstitial tissue and lymphatic capillaries in the unsatisfactory bulls (Table 1). The inverse relationship between these elements is in agreement with the hypothesis that stromal proliferation of interlobular connective tissue is a detrimental factor to the seminiferous epithelium (MEISTRICH, 1986; VOLKMANN et al., 201). Overgrowth of the interstitial tissue hinders the peritubular blood supply, resulting in hypoxia and changes in cell morphology (RAO VEERAMACHANENI et al., 1987; POP et al., 2011).

Figure 1. Box plot representing the frequency distribution (%) of changes estimated on 100 testicular seminiferous tubules according to reproductive condition in *Bos indicus* (Nellore) bulls.

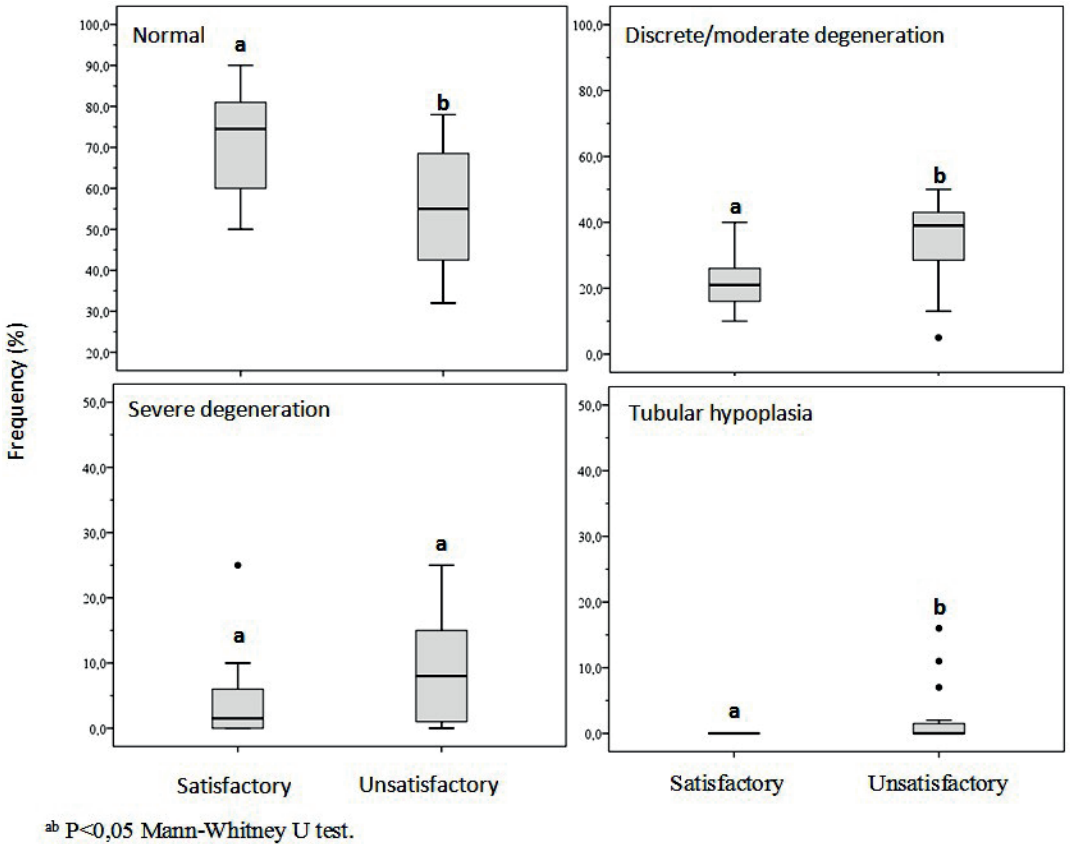


Table 1. Mean (± S.E.M.) adjusted for the percentage of stromal structures, testicular parenchyma and histomorphometric measures of the seminiferous tubules in *Bos indicus* (Nellore) bulls according to the reproductive condition.

		Satisfactory (n=10)	Unsatisfactory (n=15)	P*
Structure (%)	Seminiferous tubule	84,63±0,572[a]	81,81±0,46[b]	0,001
	Interstitial tissue	13,56±0,497[a]	15,15±0,406[b]	0,009
	Lymphatic capillary	1,35±0,148[a]	2,42±0,121[b]	0,001
	Blood vessels	0,46±0,073[a]	0,63±0,059[a]	0,058
Measurement (μm)	Epithelial height	70,0±0,54[a]	60,1±0,42[b]	0,001
	Tubular diameter	229,8±1,22[a]	231,6±1,18[a]	0,312
	Tunica propria	3,8±0,04[a]	4,4±0,03[b]	0,001

[ab] P=0,001 Student test *t* between columns, S.E.M. = standard error of the mean.

Furthermore, the increase in lymphatic capillaries from testes may be due to its hypertrophy in response to transmigration of fluids in the interstitial tissue. Variations in the relationship between lymphatic and blood capillaries may occur due to the pressure exerted by fluids throughout the testicular microvasculature, changing the patterns of the connective tissue (DAMBER; BERGH,

1992; SETCHELL; BREE, 2006). In Belgian Blue bulls, Hoflack et. al. (2008) observed a correlation between the percentage of interstitial collagen and the number of normal sperm cells (r = -0.47) and the testicular degeneration index (r = 0.63).

Disorders in human's spermatogenesis have been associated with the thickening of the lamina propria, also known as tubular sclerosis. This phenomenon is caused by an increase in the extracellular matrix due to the higher numbers of fibroblasts and myofibroblasts and increased hyaline deposition. A higher incidence of senile men with testicular degenerative changes is naturally found naturally (BUSTOS-OBREGON; HOLSTEIN, 1973; PESCE, 1987; FOLEY, 2001).

In bulls, such a condition is associated with thickening of the basal lamina and a reduction in tubular area, affecting the efficiency of spermatogenesis (RAO VEERAMACHANENI et al., 1987). This may explain the reason why the lamina propria was thicker in unsatisfactory bulls (Table 1). According to Saunders (1976), the thickness of the lamina propria in *Bos indicus* bulls with testicular degeneration can reach 5.8 mm, demonstrating a clear condition of tubular sclerosis. Volkmann et al. (2011) found that approximately 90.0% of the seminiferous tubules with thickening of the lamina propria showed defects in the germ cells and suggested that this measure can be used as testicular histopathological marker.

In this study, in addition to thickening of the lamina propria, there was a reduction in the height of the seminiferous epithelium, confirming the higher level of germ cell degeneration in the unsatisfactory group (Table 1). Tubular diameter varied from 210.4 to 254.9 μm, regardless of reproductive conditions. These values are in agreement with those found by Kumi-Diaka et al. (1983). However, the reduction in tubular density and not in diameter reiterates the histological profile where the most frequent abnormalities were of a mild to moderate degree, suggesting that these morphological changes are consistent with subfertility.

Table 2 shows quantification of the spermatogenic cycle according to the reproductive condition. Classifications were determined following the typical mitotic and meiotic cell divisions and grouping seminiferous tubules with clustered stages of cellular development (CARDOSO; GODINHO, 1983). Tubules classified as A, in the initial stages, were represented by cells with proliferative or mitotic divisions of the seminiferous epithelium. The classifications B and C represent later stages of the epithelium, grouping tubules with meiotic divisions until the differentiation of pre-spermiation spermatozoa (Figure 2). A similar classification system was proposed by Horn et al. (2003) for crossbreed bulls (*Bos indicus* × *Bos taurus*) on the basis of the stages of the spermatic cycle described by Amann (1962); the results were similar. Unsatisfactory bulls had a higher frequency ($p < 0.05$) of tubules classified as A and 5.0% less tubules classified as B and C than satisfactory group. These findings confirm the results with crossbreed bulls (HORN et al., 2003), demonstrating that cellular differentiation phases of the spermatogenic cycle are most closely related to seminal quality in both crossbreeds and Nellore bulls.

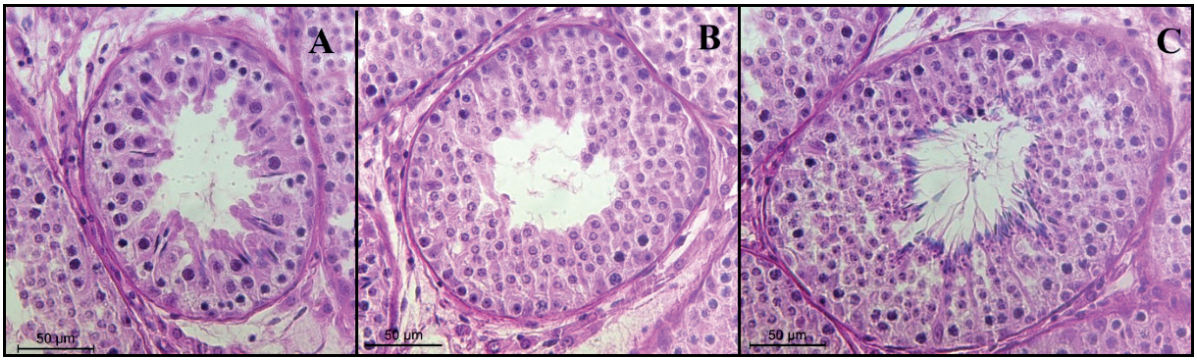
It is important to emphasize that degenerative changes in germ cells are associated with variations in histomorphometric profile, including the density of the testicular parenchyma structures. These variations are more evident in bulls with poor seminal quality. Thus, a combined analysis of qualitative and quantitative aspects of spermatogenesis is critical for inferring its efficiency according to variations in semen quality. Our findings suggest that the use of the classification system of the seminiferous epithelium by grouping in proliferation, differentiation, and post-meiosis stages must be done in combination with possible morphological alterations to the testicular parenchyma.

Table 2. Frequency (%) of spermatogenic cycle stages classification in *Bos indicus* (Nellore) bulls according to the reproductive condition.

Reproductive condition	Classification			
	A	B	C	Total
	n (%)	n (%)	n (%)	n (%)
Satisfactory (n=10)	168 (33,6)[a]	210 (42,0)[a]	122 (24,4)[a]	500 (40,0)
Unsatisfactory (n=15)	329 (43,9)[b]	276 (36,8)[b]	145 (19,3)[b]	750 (60,0)
Total	497 (39,8)	486 (38,9)	267 (21,4)	1250 (100,0)

[ab] P = 0,001 between rows; χ^2=13,64; GL=2; A= clustering of stages I,II, and III comprising the initial phases of spermatogenic cycle; B= clustering of stages IV e V; comprising the intermediary phases of spermatogenic cycle; C= clustering of stages VI, VII e VIII, comprising the final phases of spermatogenic cycle.

Figure 2. Classification of spermatogenic cycle stages in *Bos indicus* (Nellore) bulls. (A), classification A, presence of spermatogonias, primary spermatocytes and some elongated spermatids arranged in bundles. (B), segment classified as B, presence of the second meiotic division and secondary spermatocytes. (C) classification C, are found two generations of rounded and elongated spermatids and presence of sperm residual bodies.



Conclusion

Unsatisfactory bulls showed higher levels of testicular degeneration and elevated presence of hypoplasic tubules, in addition to reduced meiotic potential, when compared to satisfactory bulls for reproduction.

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