

## ORIGINAL ARTICLE

# Prevalence and Morphological Appearance of Sperm Nuclear Defects Using Feulgen Reaction in Beef Bulls

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## ABSTRACT

The objective of this study was to assess the prevalence and compare the morphological aspects of sperm nuclei abnormalities in beef bulls submitted to breeding soundness evaluation (BSE). Semen samples were collected by electroejaculation from 649 beef bulls (*Bos indicus*,  $n = 515$  and *Bos indicus* × *Bos taurus* crossbreed,  $n = 134$ ) ranging from 2 to 12 years of age. Following a clinical evaluation and semen assessment, a slide was prepared with fresh semen and stained by using the Feulgen-stain for the purpose of evaluating sperm and nuclear morphology. Abnormal sperm nuclei were classified into three categories: A, multiple vacuoles in a pouch-like formation; B, a single vacuole such as the nuclear crater/diadem defect; and C, abnormal chromatin condensation. In accordance with the BSE criteria, the animals were classified as either satisfactory or unsatisfactory potential breeders. The prevalence of nuclear defects was comparable between satisfactory bulls of both genotypes. Unsatisfactory *Bos indicus* bulls exhibited a higher frequency of A and B nuclear defect categories. A category presented an odds ratio of 7.14 (0.012) for unsatisfactory bulls. A low percentage of the C category (2.3%) was observed in both BSE classifications. Vacuoles or craters were predominantly observed in the post-acrosomal and mid-subacrosomal regions, with minimal occurrence in the apical ridge region. Our findings provide the baseline for nuclear defects in *B. indicus* and its crossbreeds. The Feulgen reaction thus enables a simple yet comprehensive analysis of nuclear sperm head defects during BSE. In addition, stain retention facilitates a practical evaluation and an accurate account of the nuclear lesion types.

## 1 | Introduction

Regardless of the classification system, sperm morphology is an important parameter for assessing semen quality, estimating fertility and predicting male reproductive function. Disturbance in spermatogenesis gives rise to morphological sperm abnormalities,

especially those associated with head shape and nuclear status (Sailer et al. 1996; Garcia-Oliveros et al. 2022). Among several types of head defects, sperm nuclear abnormalities have been investigated with different techniques for many decades and reported in association with infertility in cattle (Slizinska and Slizinski 1953; Bane and Nicander 1965; Evenson 1999; Bollwein and Malama 2023).

The positive relationship between poor sperm parameters and nuclear and DNA damage in bulls is known. Various hypotheses have been proposed as to abnormal chromatin packaging, oxidative stress and apoptosis currently present in ejaculated spermatozoa (Evenson 1999; Shaman and Ward 2006). Unfortunately, the seminal standard techniques for breeding soundness examination in bulls do not include the nuclear and chromatin status evaluation, even though some methods of evaluation are easily executed (Mello 1982; Fernandes et al. 2008).

Nuclear vacuolation occurs during spermiogenesis and may be caused by an environmental stress, although there is evidence for the heritability of this trait in some bulls (Callaghan et al. 2016; Felton-Taylor et al. 2020). In view of this uncertainty, nuclear vacuoles are given a threshold level of no more than 20% (Thundathil et al. 2001; Perry 2021). The abnormality is more commonly observed in *Bos indicus* crossbreed bulls than in *Bos taurus* breeds (Felton-Taylor et al. 2020).

The Feulgen reaction procedure has been utilised in the sperm morphology evaluation to detect and describe abnormalities in the sperm cell nucleus, such as abnormal head shapes, vacuoles and chromatin condensation that are difficult to see or recognise by conventional staining procedures. Originally, this cytochemical method was used to demonstrate DNA content by microspectrophotometry in spermatids and spermatozoa in bulls with differences in fertility (Gledhill 1966). This reaction is based on the hydrolysis of DNA by hydrochloric acid, which rapidly removes the purine bases (adenine and guanine) but leaves the sugars and phosphates of DNA intact. This hydrolysis generates an aldehyde group that can be demonstrated with Schiff reagent. The conjugated double bonds, which confer the red–purple colour to pararosaniline, are rapidly restored by the reaction of aldehydes with excess  $\text{SO}_2$  in the reagent to give an alkyl sulfonic acid that reacts with the amino groups of the leucofuchsin. Therefore, nicks or breaks of disulfide bonds are not stained and a halo or flaws in the nuclei can be observed (Barth and Oko 1989; Chieco and Derenzini 1999; Carson and Hladik 2009).

In bovine andrology, the Feulgen reaction has been used to estimate the level of chromatin abnormalities and abnormal DNA condensation. The sperm nuclei present an ample variety of forms with a predominance of clumped appearance. In addition, nuclear vacuolation may be considered a type of abnormal DNA (Barth and Oko 1989). However, the etiopathogenesis of those nuclear lesions is not completely understood. The aim of this study is to report the prevalence and to compare different morphological aspects of sperm nuclei in *B. indicus* and crossbreed beef bulls submitted to breeding soundness evaluation.

## 2 | Material and Methods

### 2.1 | Breeding Soundness Examination (BSE)

All procedures employed in this study are in accordance with the Andrological Examination Manual of the Brazilian College of Animal Reproduction, and the Brazilian Guide for the Production, Maintenance or Use of Animals in Teaching or Scientific Research Activities/National Council for the Control of Animal

Experimentation (Consea 2023). Breeding soundness examination data were collected from 2007 through 2010 at seven beef cattle farms in Campo Grande, Mato Grosso do Sul state (20°26'34" S and 54°38'47" W), Brazil. Data were available for 649 beef bulls (*Bos indicus*,  $n = 515$  and *Bos indicus* × *Bos taurus* or crossbreed,  $n = 134$ ), 2–12 years old. All bulls were submitted to physical examination, followed by a thorough examination of the reproductive tract, including the penis, prepuce, scrotum and testes, according to Fernandes and Moraes (2009). All animals were serologically negative for brucellosis and tuberculosis, and those with clinical disturbances were retired from analysis.

Semen evaluation was performed after collecting a sample via electroejaculation. The sample was then placed on a warmed (37°C) slide and covered with a cover slip. Motility (0%–100%) and vigour (0–5 scale) were immediately estimated using a light microscope at 100× magnification. Sperm morphology was evaluated under oil immersion at 1000× magnification in a phase-contrast microscope (Olympus BX 45). Morphology was classified by considering the percentage of normal spermatozoa, head defects (underdevelopment, narrow and narrow at base, abnormal contour and pyriform shape), acrosome defects (knobbed, folded, tapered, detached and vacuoles), midpiece defects (tail stump, corkscrew, broken and denuded), proximal droplet, tail defects (broken, coiled, bent tail and abaxial attachment) and loose heads. After BSE, bulls were classified by seminal attributes as satisfactory (motility > 50%, vigour > 3 and morphology > 70%), and unsatisfactory (inferior values) potential breeders. Unsatisfactory bulls were evaluated a second time before the final classification.

### 2.2 | Nuclear Sperm Morphology Evaluation

A second slide was prepared for Feulgen-stain. Briefly, smears of fresh semen were prepared and hydrolyzed in 5 N HCl for 30 min. The slides were washed and placed in a 2.0% acid solution for 15 min, washed again, and submerged in Schiff's reagent for approximately 15 min at 90°C. Preparations were then washed in running water for 5 min. When deeply stained, one or two washes in sulphite water (10%  $\text{K}_2\text{O}_5\text{S}_2$ , 1 N HCl, v/v) were performed for 30 s each.

Samples containing nuclear defects were classified according to the more prevalent defect in at least 50 microscopic fields (1000× magnification) by the Feulgen-stain preparations. Then, samples were classified into three categories: A, multiple vacuoles in the whole head or localised in the equatorial segment (classical pouch formation); B, a single vacuole or crater in any area of the head; and C, clumped nuclear material representative of abnormal chromatin condensation (Barth and Oko 1989). All defects were estimated in 200 spermatozoa/sample.

### 2.3 | Transmission Electron Microscopy

Unsatisfactory bulls ( $n = 6$ ) with more than 20.0% nuclear defects were used for ultrastructural assessment. Samples of semen (300  $\mu\text{L}$ ) were fixed in 2.5% glutaraldehyde (40 min. at room temperature) and post-fixed in a solution of 1%  $\text{OsO}_4$ , 0.8% potassium ferricyanide and 2.5 mM  $\text{CaCl}_2$  (20 min. at room temperature).

The cells were dehydrated in an ascending acetone series and embedded in Poly/Bed812 resin (Polysciences, Warrington, USA). Ultrathin sections were stained with uranyl acetate and lead citrate. A Zeiss EM10C microscope was used for examination.

Statistical analysis was performed by classifying all bulls as either satisfactory or unsatisfactory potential breeders (according to the BSE). Semen data sets were compared by ANOVA (general linear multivariate model) and expressed in a boxplot graph. Classification, genotype (*B. indicus* vs. crossbreed), and interactive effect were corrected (covariate) by age. Reproductive classifications and genotype frequencies among nuclear defects categories (no defect, A, B or C) were compared by Chi-square analysis. Next, the odds ratio of unsatisfactory bulls according to nuclear defects categories and genotype was calculated by using binomial logistic regression analysis. SPSS 23.0 (IBM) software was used for statistical analysis.

### 3 | Results

Semen traits and scrotal circumference according to reproductive classification from the BSE and genotype are summarised in Table 1. Interactive effects involving genotype (*B. indicus* vs. crossbreed) and reproductive classification were observed in motility and sperm head, including nuclear and loose head defects, as well as proximal cytoplasmic droplet, demonstrating critical variations in the crossbred bulls. The proportion of unsatisfactory bulls was

not significantly different ( $p > 0.05$ ) among *B. indicus* (129/515, 25.0%) and crossbred bulls (37/134, 27.6%).

The data analysis of the nuclear defects categories is presented in Figure 1. All three categories of nuclear defects were observed both in the satisfactory and unsatisfactory bulls. However, unsatisfactory bulls exhibited a higher frequency, particularly in categories A and B. Nevertheless, crossbred bulls ( $n = 20$ ) exhibited a high mean. Unsatisfactory bulls displayed a high mean of nuclear defects in categories A and B. Of the total number of bulls with nuclear defects identified, only 14 exhibited abnormal chromatin condensation (C category).

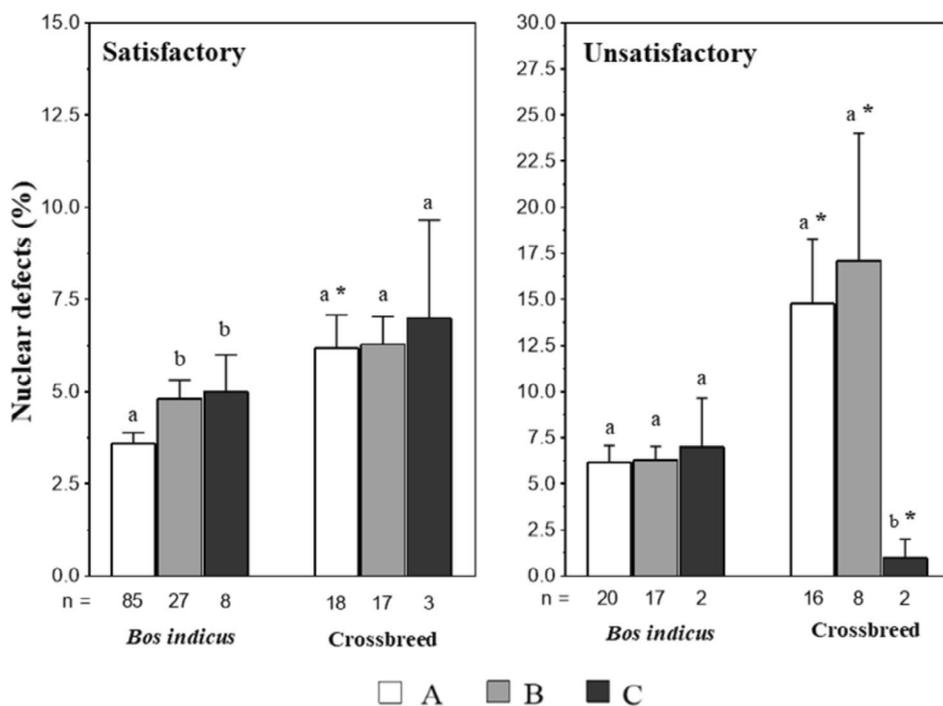
The frequencies of nuclear defects by category, reproductive classification and bull genotype are reported in Table 2. Besides the reproductive classification effect, the genotype differed for nuclear defect type A in satisfactory bulls. In unsatisfactory bulls, the effect of genotype was significant for nuclear defect type B. The odds ratio analysis of unsatisfactory bulls showed that the categories of sperm nuclear defects were significant, particularly for the A category (OR 7.14,  $p < 0.012$ ), Table 3.

Figures 2 and 3 represent the nuclear morphological defects under phase-contrast microscopy and Feulgen-stain, respectively. The Feulgen reaction allowed the identification from small nuclear vacuoles all the way to the complete abnormal chromatin condensation (total chromatin fragmentation [clumping]). Vacuole or crater defects were evidenced

**TABLE 1** | Mean ( $\pm$ S.E.M.) of semen traits and scrotal circumference according to reproductive conditions and genotype.

	Reproductive classification				G $\times$ RC
	Satisfactory		Unsatisfactory		
	<i>Bos indicus</i> ( $n = 386$ )	Crossbreed ( $n = 97$ )	<i>Bos indicus</i> ( $n = 129$ )	Crossbreed ( $n = 37$ )	
Semen traits					
Volume (mL)	4.8 $\pm$ 0.14 <sup>a</sup>	6.1 $\pm$ 0.43 <sup>b</sup>	3.5 $\pm$ 0.24 <sup>c</sup>	4.0 $\pm$ 0.71 <sup>a</sup>	0.094
Gross motility (1–4)	2.5 $\pm$ 0.16 <sup>a</sup>	1.7 $\pm$ 0.51 <sup>b</sup>	1.9 $\pm$ 0.29 <sup>b</sup>	1.9 $\pm$ 0.85 <sup>b</sup>	0.491
Motility (%)	66.8 $\pm$ 0.89 <sup>a</sup>	56.2 $\pm$ 2.74 <sup>a</sup>	52.1 $\pm$ 1.5 <sup>b</sup>	54.0 $\pm$ 4.53 <sup>b</sup>	0.026
Vigour (1–5)	3.1 $\pm$ 0.05 <sup>a</sup>	3.0 $\pm$ 0.15 <sup>a</sup>	2.8 $\pm$ 0.08 <sup>a</sup>	2.7 $\pm$ 0.25 <sup>b</sup>	0.754
Normal morphology	77.4 $\pm$ 0.58 <sup>a</sup>	79.9 $\pm$ 1.78 <sup>a</sup>	56.5 $\pm$ 1.01 <sup>b</sup>	56.4 $\pm$ 2.95 <sup>b</sup>	0.466
Sperm defect (%)					
Head	5.3 $\pm$ 0.24 <sup>a</sup>	7.5 $\pm$ 0.75 <sup>b</sup>	8.5 $\pm$ 0.42 <sup>b</sup>	11.7 $\pm$ 1.24 <sup>c</sup>	0.005
Nuclear	1.8 $\pm$ 0.27 <sup>a</sup>	2.2 $\pm$ 0.84 <sup>a</sup>	4.1 $\pm$ 0.47 <sup>b</sup>	8.1 $\pm$ 1.39 <sup>c</sup>	0.038
Acrosome	2.2 $\pm$ 0.24 <sup>a</sup>	0.4 $\pm$ 0.73 <sup>b</sup>	3.1 $\pm$ 0.41 <sup>a</sup>	0.6 $\pm$ 1.20 <sup>b</sup>	0.450
Midpiece	1.0 $\pm$ 0.16 <sup>a</sup>	0.9 $\pm$ 0.49 <sup>a</sup>	3.6 $\pm$ 0.28 <sup>b</sup>	2.3 $\pm$ 0.81 <sup>b</sup>	0.270
Proximal droplet	0.6 $\pm$ 0.17 <sup>a</sup>	0.1 $\pm$ 0.53 <sup>a</sup>	2.6 $\pm$ 0.030 <sup>b</sup>	1.3 $\pm$ 0.88 <sup>b</sup>	0.039
Loose head	4.0 $\pm$ 0.38 <sup>a</sup>	3.3 $\pm$ 1.18 <sup>b</sup>	11.2 $\pm$ 0.67 <sup>c</sup>	5.2 $\pm$ 1.95 <sup>a</sup>	0.028
Tail	6.8 $\pm$ 0.39 <sup>a</sup>	4.4 $\pm$ 1.20 <sup>a</sup>	10.6 $\pm$ 0.68 <sup>b</sup>	7.5 $\pm$ 2.00 <sup>a</sup>	0.047
Scrotal circumference (cm)	39.4 $\pm$ 1.76 <sup>a</sup>	37.5 $\pm$ 3.50 <sup>a</sup>	38.5 $\pm$ 3.00 <sup>a</sup>	36.3 $\pm$ 5.67 <sup>a</sup>	0.968

Note: G  $\times$  RC, interactive effect between genotype and reproductive condition. <sup>a,b</sup>Within a row means without a common superscript differ ( $p < 0.05$ ).



**FIGURE 1** | Mean ( $\pm$ SEM) of nuclear defects according to the breeding soundness classification, bull genotype and abnormal nuclear categories: (A) multiple small vacuoles in a pouch formation; (B) single vacuole or crater defect in the head area; and (C) abnormal chromatin condensation. (n), number of bulls. Different letters represent a significant difference ( $p < 0.05$ ) between reproductive classifications within genotype; an asterisk denotes  $p < 0.05$  between genotype for the same class.

**TABLE 2** | Frequency by type of nuclear defect according to the effects of reproductive category and genotype in beef bulls.

Sperm nuclear defect	Reproductive classification			
	Satisfactory		Unsatisfactory	
	Bos indicus (n = 386)	Crossbreed (n = 97)	Bos indicus (n = 129)	Crossbreed (n = 37)
A	85 (17.6)	18 (3.7)*	20 (12.0)	16 (9.6)
B	27 (5.6)	17 (3.5)	17 (10.2)	8 (4.8)*
C	8 (1.7)	3 (0.6)	2 (1.2)	2 (1.2)
Total	120 (31)	38 (39.2)	39 (30.2)	26 (70.3)*

Note: A, multiple vacuoles; B, simple vacuole (crater defect); C, abnormal chromatin condensation. Satisfactory,  $\chi^2 = 11.250$  ( $p < 0.010$ ); Unsatisfactory,  $\chi^2 = 19.5494$  ( $p < 0.001$ ).

\* $p < 0.05$  between genotype.

**TABLE 3** | Odds ratio<sup>a</sup> estimation for unsatisfactory bulls according to the type of nuclear defect and genotype.

Sperm nuclear defect	Coefficient	SE	Wald	p	OR	CI 95%
A	1.966 ( $X_1$ )	0.966	4.146	0.012	7.14	1.07–47.41
B	1121 ( $X_2$ )	0.947	1.401	0.042	3.07	0.47–19.63
C	1196 ( $X_3$ )	0.961	1.497	0.123	3.22	0.48–21.19
Genotype	0.620 ( $X_4$ )	0.240	0.066	0.797	1.06	0.66–1.25

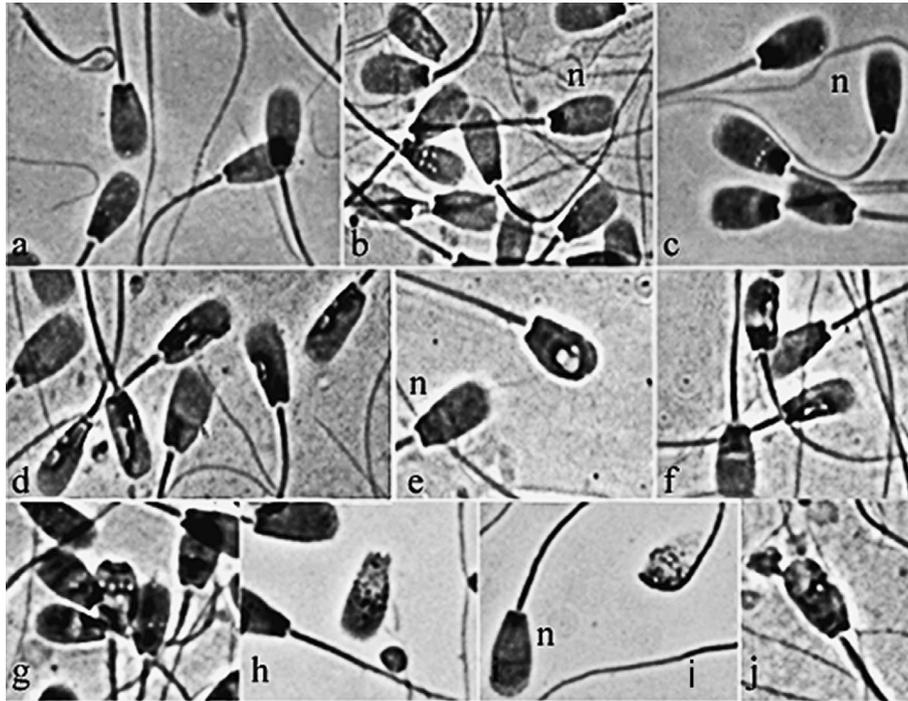
Note:  $[\text{LogitPi} = -3.249 + (X_1) + (X_2) + (X_3) + (X_4)]$ ;  $\chi^2 = 57.057$ ; GL 3;  $p < 0.001$ .

Abbreviations: CI, confidence interval; OR, odds ratio; SE, standard error.

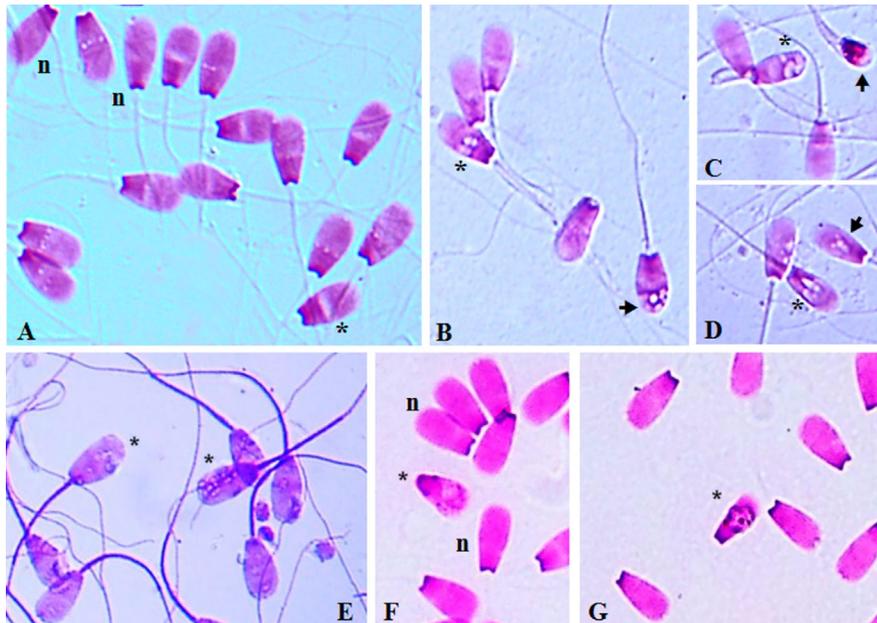
<sup>a</sup>Binomial logistic regression analysis.

with a predominance of post acrosomal margin and mid-subacrosomal region; few craters were seen in the apical ridge region. In agreement with observations reported by Barth and

Oko (1989), DNA fragmentation was observed as a coarsely clumped chromatin. Many normal morphological heads presented a central-halo, clear and variable in size. Representative



**FIGURE 2** | Photomicrographs of sperm nuclei evaluated in phase-contrast microscopy. (a–c) Small vacuoles scattered in the nuclear area; multiple vacuoles are localized in the equatorial segment representing the classical pouch formation; n denotes normal nucleus. (d–f) Severe vacuolation in the nuclear area with modifications of head structure and a shape like crater/diadem defects and their sequelae. Note the complete rupture of nuclear membrane with formation of a clear hole or crater in the centre of the nucleus (e). (g–j) Representative images of various aspects of abnormal chromatin condensation: (g) two nuclei vacuolized with large central holes, (h) and (i), clumping of nuclear chromatin; (j) nucleus with vacuole and chromatin partially condensed. Feulgen reaction, 1000 $\times$ .



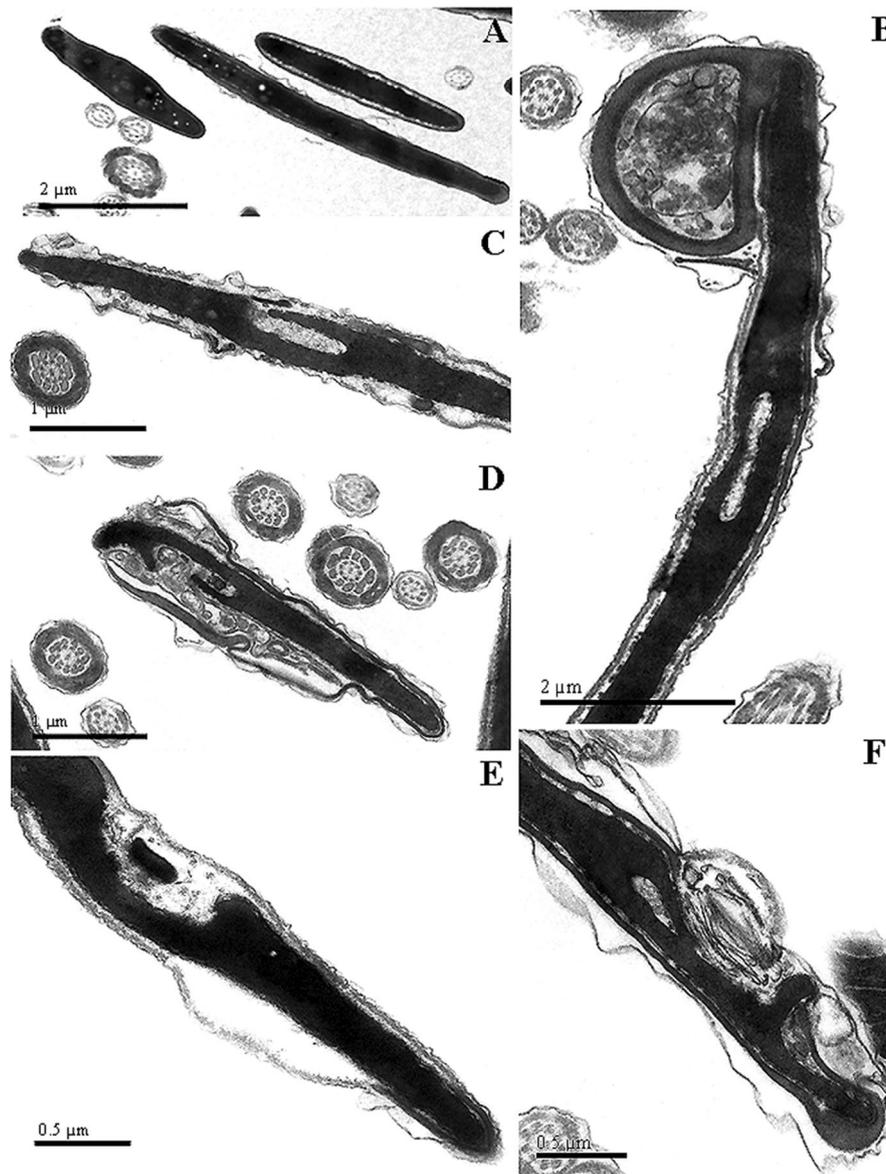
**FIGURE 3** | Phase-contrast micrographs of nuclear defects stained by Feulgen reaction. (A) Small vacuoles scattered in the nuclear area; multiple vacuoles are localised in the equatorial segment representing the classical pouch formation; nuclear decondensation (asterisk); class a. (B–D) Severe vacuolization in the nuclei with modifications of head structure and a shape like crater/diadem defects. (B) Complete rupture of nuclear membrane with formation of a clear hole or crater in the centre of the nucleus (asterisk) and apical region (arrow). (C) Crater defect with abnormal shape of nucleus (asterisk) and microcephalic head (arrow). (D) Different types of nuclear defects; elongated crater in central area (asterisk) and multiple vacuoles-type (arrow). (E–G) Aspects of abnormal chromatin condensation. (E) Nuclei with initial chromatin fragmentation and different levels of nuclear envelope rupture (asterisk). (F) Abnormal heads (asterisk) with partially damaged chromatin in comparison of normal nuclei (n). (G) A small nuclear sperm with poor chromatin condensation (asterisk). 1000 $\times$  magnification.

transmission electron micrographs images of r abnormal nuclei are shown in Figure 4.

#### 4 | Discussion

The fertilising ability of the bulls entering breeding programmes is critical for determining the reproductive performance of a herd, irrespective of the fertility status of the cows. Sub-fertility and infertility occur in bulls due to a variety of causes following problems in spermatogenesis or due to the inability to mount. Poor seminal quality includes a wide variety of sperm abnormalities, particularly those relative to nuclear shape, size and structure (Ostermeier et al. 2001; Fernandes et al. 2008).

Phase-contrast microscopy is usually utilised to assess sperm morphology; however, the combination with Feulgen stain enables a more detailed evaluation. The process of acid exposure is stressful to the defective chromatin, which normal, intact DNA tolerates. The acid removes the purines from the DNA and exposes the aldehyde groups for Schiff's reagent to bind. Thus, fine points can be observed, leading to more confidence in identifying abnormal sperm cell nuclei (Chieco and Derenzini 1999). Ferrari et al. (2018) evaluated chromatin structure in bull semen and found differences between normal, large and diploid nuclei with Feulgen stain. The area and volume of enlarged sperm nuclei were greater than those of normal-sized nuclei, whereas the mean and maximal absorbance were lower, suggesting incomplete and/or defective chromatin condensation in large nuclei.



**FIGURE 4** | Sagittal sections through the heads of bovine spermatozoa as seen by transmission electron microscopy. (A) Spermatozoa nuclei presenting inclusions of varied size. (B) Extensive vacuolar area in the nucleus with ruptured chromatin and oedema of the outer acrosomal membrane. (C) Nucleus with a central inclusion and abnormal acrosome with vacuole formation; note thickening of the apical region of the acrosomal membranes. (D) Nuclear inclusion associated with partial chromatin lysis. (E) Nuclear vacuole with retention of membranes and myeloid inclusions. (F) Abnormal acrosome associated with a very large vacuole containing lamellar membrane inclusions.

In the present study, the Feulgen reaction stained the three classes of nuclei with different intensity, allowing them to be recognised clearly when using light microscopy. Also, chemical and structural modifications of chromatin, a DNA–protein complex, may disturb chromatin condensation during spermiogenesis, which could be the origin of abnormalities such as macrocephaly, associated with nuclear vacuoles, diploidy and chromatin condensation defects (Revay et al. 2009). These defective nuclei are usually wider and longer than the normal ones and are associated with low fertility or even complete infertility (Barth and Oko 1989; Ferrari et al. 1998; Chemes and Rawe 2003; Guthauser et al. 2016).

Of the 649 bulls examined, 483 (74.4%) were classified as satisfactory, and 166 (25.6%) as unsatisfactory. The unsatisfactory proportion might appear high, but considering the wide range of age (2–12 years old), genotype, management and other factors, this is probably a suitable reflection of breeding soundness examination in the Midwest region, a savannah-like ecosystem. These finds are similar to those reported in other regions of Brazil (Moraes et al. 1998; Vale-Filho et al. 1996). Unfortunately, there are few reports on the prevalence of nuclear defects and abnormal chromatin condensation in beef bulls in Brazil, a gap that the present study helps to fill.

As expected, the means of nuclear defects in all unsatisfactory bulls were high; however, the means of defects of type A and B were higher in the unsatisfactory crossbreed bulls than in *B. indicus* ones. One possible explanation is that perhaps the infusion of angus blood takes away some of the indicus ability to adapt to heat stress, making the crossbreeds more prone to heat-induced spermatogenic defects. These results demonstrate that bulls with acceptable semen quality can present low percentages of lesions in the nuclei/chromatin, probably without compromising fertility. On the other hand, nuclear defects in unsatisfactory bulls may present high prevalence with serious impediment to fertility. Here, the mean percentages observed are similar to previous studies in which the prevalence of affected spermatozoa ranged from 3% to 70%, in young and older *B. taurus* bulls used in artificial insemination programs (Hrudka and Zibrin 1964; Jiranek and Rob 1971; Coulter et al. 1978).

The pathogenesis of bovine sperm nuclear vacuolation is not widely recognised. Vacuoles and chromatin lesions are supposedly formed spontaneously around the cap phase of spermiogenesis at the beginning of nucleoplasm condensation and nuclear remodelling (Barth and Oko 1989; Dobrinski et al. 1994). For instance, rolled nuclei and irregular distribution of nucleoplasm originate the classical pouch formation (Miller et al. 1982), in the present study classified as A type (Figure 2A–C). There is evidence that these defects are confined only to the nuclear envelope (Escalier et al. 2003). In the studied bulls, the vacuoles varied in size and number among individuals, and it is possible that they reflect the conditions of nuclear maturity or immaturity. This aspect is particularly observed in humans where the vacuoles are in contact with the nuclear matrix (Toshimori 2009). Therefore, these nuclear lesions can be considered frequent, with a prevalence of affected spermatozoa ranging from 0% to 8% in *Bos indicus* and 0% to 15% in crossbred satisfactory bulls. Furthermore, our results demonstrate that these abnormalities are more likely

to occur in crossbreed, and seven times more likely to occur in bulls unsatisfactory for breeding, followed by a simple vacuole or crater defect and the complete abnormal chromatin condensation evaluated by the Feulgen-stain technique.

The B and C category lesions were more prevalent in unsatisfactory bulls. The overall incidence, however, was low. Both categories are characterised by defect types originating from profound disruption of spermiogenesis. The vacuoles found in the B category are larger craters enclosed in the sperm head. The B and C category lesions most likely originate from significant disruption of spermiogenesis. The vacuoles found in the B category are larger craters enclosed within the sperm head and are often observed in different head regions, compared to those reported by Heath (1982). These nuclear lesions contain excess cytoplasmic components that are eliminated by the Sertoli cell during spermiogenesis under normal conditions (Toshimori 2009). In humans, this stage of nuclear degeneration involves DNA strands disrupted and enclosed by unusually large amounts of cytoplasmic components of the nuclear matrix (Holstein et al. 1988). Additionally, apoptotic forms visualised in germ cells during spermatogenesis present abnormal nuclear morphology and a vacuolar pattern rich in amorphous material (Baccetti et al. 1996). On the other hand, the C category was identified in a smaller bulls population that usually also presented nuclei with greater vacuoles (Figure 2J,G) and rare ghost nuclei according to Barth and Oko (1989). In fact, nuclear vacuolation may be considered a type of abnormal DNA condensation. However, the appearance is typical with clumping formation distributed in the whole nuclear area. This condition occurs when spermatogenesis is severely affected and the male becomes infertile (Dobrinski et al. 1994; Fernandes et al. 2008). Finally, the B and C type nuclear defects were associated with head defects such as the narrow head, narrow at the base, abnormal contour and elongated head associated with reduced motility, suggesting an ongoing testicular degenerative process.

The Feulgen reaction is an easy and inexpensive procedure well known in biology laboratories. This technique allows an accurate analysis of the nuclear and sperm head appearance, especially under phase-contrast microscopy, and has been used to recognise diploid and large nuclei using light microscopy in horses (Spirito et al. 2011), boars (González et al. 2013) and llamas (Giuliano et al. 2018). To the extent of our knowledge, this is the first study to systematise nuclear defects in a significant number of bulls under field conditions and provide the baseline for nuclear defects in *B. indicus* and its crossbreeds. The Feulgen reaction has permitted us to perform a simple yet comprehensive analysis of nuclear sperm head defects in routine semen examination. Furthermore, stain retention facilitates practical assessment and accurate reporting of nuclear lesion types.

#### Author Contributions

All authors reviewed and approved the revision.

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## Conflicts of Interest

The authors declare no conflicts of interest.

## Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author.

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