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Sperm morphometry and chromatin condensation in Nelore bulls of different ages and their effects on IVF

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ABSTRACT

The aim of this study was to evaluate the chromatin packing and sperm head morphometry of cryopreserved semen of Nelore bulls (*Bos taurus indicus*) of different ages. Furthermore, the influence of the degree of chromatin compaction on *in vitro* embryo production (IVP) was investigated. Forty bulls were divided into three groups: young (1.8–2 years), adult (3.5–7 years), and senile (8–14.3 years). The ejaculates were frozen according to standards established by the Artificial Insemination Center located in the Southeast of Brazil. Toluidine blue staining was used for simultaneous evaluation of the sperm chromatin and sperm head morphometry. Chromomycin A3 (CMA3) was applied to analyze sperm protamination and IVP for embryonic development. Spermatozoa of young bulls presented higher values for area (A, pixels), perimeter (P, pixels), and width (W, pixels) compared to adults and senile (young: $A = 1848.5 \pm 119.79$, $P = 10.23 \pm 0.29$, and $W = 1.95 \pm 0.1$; adults: $A = 1672.9 \pm 104.46$, $P = 9.86 \pm 0.33$, and $W = 1.81 \pm 0.06$; senile: $A = 1723.1 \pm 124.41$, $P = 9.97 \pm 0.33$, and $W = 1.83 \pm 0.09$; $P < 0.0001$) and showed higher protamination deficiency when analyzed by CMA3 (young: 1.57 ± 0.76; adults: 1.09 ± 0.63, and senile: 0.90 ± 0.59; $P < 0.05$). Likewise, variables of sperm head size (A, P, and W) and protamination assessed by CMA3 showed negative correlation with age and positive correlation with ellipticity, evaluated by toluidine blue method ($P < 0.05$). Sperm head area was larger in spermatozoa presenting chromatin instabilities than spermatozoa without chromatin alteration ($P < 0.0001$). There was no difference in IVP when using semen with larger or smaller portions of spermatozoa with chromatin instabilities, indicating that the proportion of sperm with abnormal chromatin compaction (4%–16.15%) did not interfere with early embryonic development. From our results, it can be concluded that sperm of young Nelore bulls have larger heads compared to adults and senile due to reduced protamine content when evaluated by CMA3 and higher proportion of major sperm defects assessed by differential interference contrast microscopy.

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1. Introduction

Embryonic mortality in cattle is pointed out as the main source of economic loss for livestock producers [1]. Embryo survival is a major factor affecting production and economic efficiency in all systems of ruminant milk and meat production. In Brazil, the embryonic mortality in cattle leads to a loss of 350 to 850 million US$/year [2]. Semen analysis, a primary method of fertility diagnosis, evaluates sperm count, motility, morphology, plasma membrane, and acrosomal membrane integrity. However, modifications in sperm chromatin are usually not taken into account [3]. Spermatozoa containing chromatin instabilities are able to fertilize oocytes both *in vivo* and *in vitro*; however, the defect may persist throughout the embryonic period, inducing apoptosis, embryonic disruption, and abortion [4,5].

Previous to meiosis, the chromatin in the spermatocyte nucleus is diffusely organized similarly to the nucleus of all somatic cells. The predominant proteins in this phase are histones, which increase their volume [6,7]. When spermiogenesis is initiated, protamines become most abundant in mammalian sperm nucleus and allow high organization, condensation, and compaction [8,9].

The head of mammalian spermatozoa consists almost entirely of chromatin. In case of changes in chromatin structure, morphologic abnormalities are expected [3,10,11]. However, some studies have shown that sperm containing abnormal chromatin condensation do not necessarily present morphologic changes, requiring morphologic analysis followed by evaluation of the internal structure [12,13].

Recent studies regarding the compaction of chromatin related to sperm head morphometry in cattle do not compare different age groups [11,14–16]. The aim of this study was to investigate the relationship between chromatin condensation and sperm morphometry in young, adult, and senile Nelore bulls and its influence on fertility.

2. Material and methods

2.1. Animals and experimental design

A total of 40 healthy Nelore bulls, kept on native pasture (*Cynodon plectostachyus*) with dietary supplementation to fulfill energy balance, were selected from an Artificial Insemination Center located in Southeastern Brazil (21°09′56.58″S/048° 02′ 25.13″W). The ejaculates were obtained by artificial vagina, routinely tested and classified as normospermia and frozen in 0.25-mL straws according to standard procedures established by Artificial Insemination Center and stored in liquid nitrogen until evaluation. Three groups of bulls were evaluated, with three ejaculates per animal: young group (from 1.8- to 2-years old, n = 9), adult group (from 3.5- to 7-years old, n = 19), and senile group (from 8- to 14.3-years old, n = 12). All evaluations in this study were performed with cryopreserved semen samples.

2.2. Semen evaluation

2.2.1. Conventional physical tests

Semen thawing was performed at 35 °C for 20 seconds. Postthaw semen evaluation included progressively motile spermatozoa (%), sperm concentration (Neubauer chamber), and spermatozoa with morphologic abnormalities (%, differential interference contrast microscopy [Olympus BX61] in a humid chamber, × 1000) [17].

Three ejaculates were collected from each bull using an artificial vagina according to a regular twice-a-week collection schedule and evaluated according to standard procedures for volume (mL), sperm concentration (10^6/mL, photometry), gross motility (zero to five), progressively motile spermatozoa (%) (subjective estimation using phase contrast microscopy, × 200), and spermatozoa with morphologic defects. For evaluation of sperm morphology, spermatozoa were fixed in a stock solution of buffered formol saline [18]. Sperm alterations were classified into major defects (i.e., primary acrosome defects, proximal droplets, abnormal loose heads, abnormal head contour, abnormal midpiece, nuclear vacuoles, double forms, and dag defect) and minor defects, a total of 200 cells were examined per sample [19,20].

2.2.2. Sperm head morphometry and chromatin condensation evaluation with toluidine blue

Gray-level digital images of a minimum of 100 spermatozoa per smear were obtained (light microscopy, Olympus BX 61 coupled to an DP-71 Olympus camera, × 100 magnification), and the average intensities of the gray-levels per head in each image were determined using software programs developed in the SCILAB environment, according to the protocol established by Beletti and Costa [21].

The reference for the normal spermatozoa staining was performed by an automatic selection of six heads in each smear and considering the mean value of the pixel values of these heads. Heads presenting the highest average pixel values and homogeneousness were selected. Next, for each image, the difference between the average value of standard heads and the value of each head examined was determined. Finally, the coefficient of variation (%) of gray level and the percentage difference in the degree of ash (% diff) was calculated. Sperm heads with difference greater than 2.0% and/or gray level to the degree of ash greater than 5.0 were considered to have abnormal chromatin structure [22].

Morphometric variables evaluated by this technique by programs developed in SCILAB were area (A), perimeter (P), width (W), length (L), length/width ratio (L/W), ellipticity (E), shape factor (SF), Fourier descriptors with amplitude of zero to two (F0, F1, and F2), lateral symmetry (LS), and anteroposterior symmetry (APS) [21].

A, P, W, and L have become standard measures; L/W and E are derived from the previous basic measures. Ellipticity is described as measure of elongation of the head contour, normalized such that $-1<$ and <1. SF is obtained from the basic measures and indicates the deviation of the head contour from smooth ellipse [21].

2.2.3. Evaluation of sperm protamination with chromomycin A3 (CMA3)

For evaluation of sperm protamination, the CMA3 fluorescent dye (Sigma, St. Louis, MO, USA) was used. The staining protocol for flow cytometry has been developed