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Sperm chromatin alterations in fertile and subfertile bulls

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ABSTRACT

Alterations in sperm chromatin have been related with subfertility in several mammals. In this study, chromatin alteration types (Base, Basal half, Central axis, Dispersed, and Whole) were assessed by toluidine blue (TB) staining, 6-diamidino-2-fenilindole (DAPI) and anti-protamine 1 antibody (anti-PR1) labeling in sperm samples of fertile and subfertile bulls. Semen samples were obtained from bulls kept in Artificial Insemination Center (fertile bulls) or from bulls subjected to scrotal insulation (subfertile bulls). The percentage of chromatin alterations identified by TB was similar ($P > 0.05$) in semen samples of fertile and subfertile bulls. In contrast, a greater ($P < 0.01$) chromatin decondensation and heterogeneity were recorded in semen samples of subfertile bulls. In DAPI and anti-PR1 methods, the subfertile bulls samples had a higher ($P < 0.05$) percentage of alteration in the base as well as overall chromatin alterations ($P < 0.05$). Moreover, the chromatin alterations recorded with TB, DAPI, and anti-PR1 were compared in semen samples of fertile and subfertile bulls. In fertile bulls, the overall chromatin alterations were similar ($P > 0.05$) among the methods In contrast, semen samples of subfertile bulls had a higher ($P < 0.05$) percentage of overall chromatin alterations when labeled with DAPI. In conclusion, our findings shown that all dye tested had specific sperm stainability and can be feasible to monitor subfertility condition in bulls. Also, different chromatin alteration types in sperm samples of fertile and suberftile bulls were recorded.

1. Introduction

Routinely, semen quality is determined according to concentration, motility, and morphology of spermatozoa which give a degree of subjectivity in the results [3]. Furthermore, these conventional approaches are poor predictors of male fertility, suggesting that the causes of low fertility may be related to damages in sperm membrane or chromatin [27].

Chromatin of mature spermatozoa differs in structure and composition from chromatin of somatic cells [18]. During the spermatogenesis, the histones are replaced by protamines that have a high ability to package the sperm DNA [8]. However, throughout the chromatin remodeling intrinsic factors such as apoptosis, reactive oxygen species, and protamine deficiencies can induces DNA damage leading to a poor chromatin condensation and, consequently, male subfertility [21]. Over the past two decades new techniques have been developed to assess the role of chromatin components on spermiogenesis and fertility. Thus, sperm chromatin integrity can be assessed by different approaches, such as toluidine blue (TB) [3], acridine orange [30], aniline blue [13], comet assay [26], TUNEL assay [29], sperm chromatin structure assay (SCSA) [10], chromomycin A3 test (CMA3) [24], sperm chromatin dispersion test (SCD) [11], and DNA breakage detection-fluorescence in situ hybridization test (DBD-FISH) [7].

Even so, the researchers have using complex methodologies [32] or a combination of multiparametric tests [31] to explore the relationship between sperm DNA damage and fertility. However, only a few of these approaches are used in livestock species mainly because the cost. Thus, efforts to develop techniques of chromatin assessment that allow the identification of detailed chromatin alterations in fertile and subfertile bulls are still required. Therefore, the aim of this study was to apply different methods for chromatin assessment (toluidine blue – TB, 6-diamidino-2-fenilindole – DAPI, and anti-protamine 1 antibody – anti-PR1) to identify chromatin alteration types in sperm samples of fertile and subfertile bulls.

2. Material and methods

2.1. Semen samples and experimental design

Experimental procedures were performed according to the

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Institutional Animal Care and Use Committee of the Federal University of Uberlândia (protocol #017-12). Fertile samples (n = 8) were obtained from three bulls kept on an Artificial Insemination Center. All samples were submitted to quality control (sperm features after thawing: > 50% motility and < 10% total abnormalities) according to standard procedures stablished by the Brazilian Ministry of Agriculture, Livestock and Supply.

Subfertile semen samples (n = 8) were obtained from bulls subjected to 72-h scrotal insulation to induce heat stress, as described in our previous study [16]. Briefly, ejaculates were collected at Day 0 (preinsulation period) and once weekly at Days 7, 14, 21, 28, 35, 42, 49, and 56 after scrotal insulation. Then, the semen was frozen and used for in vitro production of embryos. In the present study, only semen samples after Day 28 of scrotal insulation were used due the lower cleavage and blastocyst rates, as reported [16]. The content of each sperm straw (fertile and subfertile bulls) was evaluated by different methods for chromatin assessment as follows: TB, anti-PR1, and DAPI.

2.2. TB staining

Semen samples were thawed (37 °C for one minute) and subjected into a smear preparation, as previously described [2]. Briefly, the smears were fixed in ethanol acetic acid (3:1, v/v) for 1 min followed by 70% ethanol (3 min). Then, the smears were acid hydrolyzed (4N hydrochloric acid) for 25 min, washed in distilled water and dried at room temperature. The slides were stained with a droplet of TB 0.025% (w/v) prepared at pH 4.0 in a sodium citric acid-phosphate buffer (McIlvaine buffer), and then covered with a coverslip. After 3 min, the images were obtained using light microscope at magnification 1000 × (Leica DM500) and an image capture system (Leica LAS EZ software version 1.8.1).

2.3. Immunocytochemistry and fluorescence microscopy

For this analysis, the semen samples were placed in a 2 mL vial and washed three times with sodium phosphate buffer (PBS) by centrifugation (100*g* for 10 min) at room temperature. Subsequently, the pellet was resuspended and incubated in 2 mL of solution containing 25 mM Tris buffer, 10 mM dithiothreitol (DTT), 0.5% (v/v) Triton X-100, and 100 IU of heparin sodium (Hepamax) for 60 min. After incubation, the pellet was washed in PBS by centrifugation, fixed in 4% formaldehyde (v/v) for 60 min, and washed three times in PBS. Finally, the pellet was resuspended in 1 mL of PBS and twenty microliters were applied in slides for immunofluorescence that dry at room temperature.

The mouse monoclonal anti-protamine 1 antibody (Hup 1N, Briar Patch Biosciences LLC) was diluted 1:100 in their blocking buffer [PBS, 0.15% gelatin (w/v), 0.1% azide (w/v) and 0.1% saponin (w/v)], placed on slides, and incubated overnight at 4 °C. Subsequently, the slides were washed in PBS and the secondary antibody (1:100) was applied (Alexa Fluor 488 rabbit anti-mouse IgG, Life Technologies) with DAPI (1:500) for 60 min. After washing, glycerin with 1,4-Phenylenediamine (PPD) was added to the slide and immediately covered with a coverslip. Fluorescence images were acquired using a Zeiss LSM 710 confocal microscope (Carl Zeiss, Inc., Thornwood, NY, USA) at magnification 630 × under oil immersion. A laser ray at 488 nm was used to identify the Alexa Fluor 488 fluorophore (500 nm exposure and 580 nm emission), whereas a laser ray at 405 nm was used to identify the DAPI fluorophore (400 nm exposure and 480 nm emission).

2.4. Computational image analysis

2.4.1. Steps to assess the chromatin alteration types

Chromatin alteration types were assessed in sperm heads stained with TB (Fig. 1B–G) and labeled with DAPI (Fig. 2A–E) and anti-PR1 (Fig. 2F–J). The images obtained by light and confocal microscopes were processed and evaluated using algorithms developed in the MATLAB environment. Firstly, the software performed the segmentation of sperm heads (at least 300 heads per sample were evaluated). Subsequently, the pixel levels within the sperm head in each image were used to determine the chromatin alteration types. To set these regions, the following procedures were applied:

(1) Mathematical morphology technique (erosion operation) was used to discard the pixel on the perimeter of the sperm head (a circular structure element of radius 2 was used); (2) The mean pixel intensity of each sperm head was computed, and the six highest values were selected; (3) The average of the six highest values was used to define a reference value '*x*' in pixels unit. Pixels values greater than '*x*' indicates altered region in the head; (4) Image thresholding was applied in each head using '*x*' as a reference value; (5) Thereafter, the result image was smoothed using a Gaussian filter (sigma = 2; radius = 2.5) to reduce noise and better delimited the altered region; (6) Finally, for visual analysis a thin border was drawn to defined the regions of interest.

2.4.2. Chromatin alteration types

The chromatin alterations (Fig. 1A) were classified as previously described [1], with some modifications as follows: Base (chromatin alteration up to the basal third of sperm head); Basal half (chromatin alteration up to two thirds of the sperm head); Central axis (chromatin alteration in the central axis without achieve the sides of the sperm head); Dispersed (scattered chromatin alteration points in the sperm head); and Whole (sperm head chromatin totally altered). All evaluations were performed by a single operator.

2.4.3. Steps to assess the chromatin decondensation and heterogeneity

The TB stained images that were previously targeted were also evaluated to determine the chromatin decondensation and heterogeneity (at least 300 sperm heads per sample were evaluated) [16]. The following steps were conducted:

(1) An algorithm developed (MATLAB) automatically selected the six heads that were the most compact and homogeneous in each sample; (2) The average of the pixel value of these heads was used as the reference value for the normal staining sperm (standard head); (3) Then, for each image the difference between the average value of the standard heads and the average value of each head analyzed was determined; (4) This difference was transformed into a percentage of the average value and used as a quantitative indicator of sperm chromatin decondensation; (5) Moreover, sperm chromatin heterogeneity was determined by the coefficient of variation of gray levels for each sperm head.

2.5. Statistical analysis

All statistical analyses were performed using Sigma Plot 11 (Systat Software Inc., USA). The percentage of chromatin alteration, decondensation, and heterogeneity between fertile and subfertile semen samples were evaluated by Wilcoxon signed test, whereas the Kruskal–Wallis test was used to compare the percentage of chromatin alteration recorded among the methods. Data are presented as mean percentage (± SEM), and the results were considered different when $P < 0.05$ (two-sided test). Probability values > 0.05 and < 0.1 indicated that a difference approached significance.

3. Results

The mean percentage of chromatin alterations identified by TB in semen samples of fertile and subfertile bulls are shown (Table 1). Overall, the chromatin alteration did not differ ($P > 0.05$) between fertile and subfertile bulls. In contrast, a greater ($P < 0.01$) chromatin decondensation and heterogeneity were recorded in semen samples of subfertile bulls (Table 2).

When the sperm samples were evaluated by DAPI (Table 3), the

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