Theriogenology 86 (2016) 924–933

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Selected sperm traits are simultaneously altered after scrotal heat stress and play specific roles in *in vitro* fertilization and embryonic development

Aline C. Lucio, Benner G. Alves, Kele A. Alves, Muller C. Martins, Lucas S. Braga, Luisa Miglio, Bruna G. Alves, Thiago H. Silva, José O. Jacomini, Marcelo E. Beletti*

Laboratory of Biology of Reproduction, Institute of Biomedical Sciences, Federal University of Uberlândia, Uberlândia, MG, Brazil

ARTICLE INFO

Article history:
Received 5 November 2015
Received in revised form 10 March 2016
Accepted 10 March 2016

Keywords:
Bovine
Chromatin integrity
In vitro fertilization
Sperm-head morphometry

ABSTRACT

Improvements in the estimation of male fertility indicators require advances in laboratory tests for sperm assessment. The aims of the present work were (1) to apply a multivariate analysis to examine sperm set of alterations and interactions and (2) to evaluate the importance of sperm parameters on the outcome of standard IVF and embryonic development. Bulls (n = 3) were subjected to scrotal insulation, and ejaculates were collected before (preinsulation = Day 0) and through 56 days (Days 7, 14, 21, 28, 35, 42, 49, and 56) of the experimental period. Sperm head morphometry and chromatin variables were assessed by a computational image analysis, and IVF was performed. Scrotal heat stress induced alterations in all evaluated sperm head features, as well as cleavage and blastocyst rates. A principal component analysis revealed three main components (factors) that represented almost 89% of the cumulative variance. In addition, an association of factor scores with cleavage (factor 1) and blastocyst (factor 3) rates was observed. In conclusion, several sperm traits were simultaneously altered as a result of a thermal insult. These sperm traits likely play specific roles in IVF and embryonic development.

1. Introduction

The structural and functional integrity of spermatozoa have been analyzed by several laboratory techniques to determine the potential relationship between semen parameters and fertility [1–5]. As a complement of these techniques, the scrotal insulation model is a useful experimental tool that has the potential to be applied in several fields of research [6–9]. Thus, the thermal insult produces remarkable variety of morphological sperm alterations which can be evaluated and associated to male reproductive performance [10–12].

Traditionally, computer-aided methods have been used to analyze primary (area, perimeter, width, and length) and derived measurements (ellipticity, shape factor, and Fourier harmonics) to provide information on sperm-head dimensions [13–16]. Moreover, chromatin integrity analyses [17,18] have also been applied and reported a significant association with embryo quality [19,20], embryonic development [21], and pregnancy rates [22]. However, improvements in the estimation of male fertility indicators require advances in the development of laboratory tests for sperm assessment [23], better conditions for fertility evaluation [24], and use of elaborate statistical techniques [25].

It is becoming increasingly evident that conventional approaches to sperm quality assessments, which consider only specific spermatozoal traits [26] or evaluate male fertility based just on average values of sperm parameters after single functional tests [27], are poor predictors of fertility [28,29]. As a result, researchers have sought

* Corresponding author. Tel./fax: +55-34-3225-8482.
E-mail address: mebeletti@ufu.br (M.E. Beletti).

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http://dx.doi.org/10.1016/j.theriogenology.2016.03.015

methods to incorporate multiparametric assessments of sperm-head traits in a clinically useful manner [30–32]. Previous reports have used a combination of experimental techniques and multivariate statistical analyses (e.g., principal component analysis [PCA], exploratory factor analysis, and clustering analysis) to reduce several variables to a few factors and explore the relationships between different sperm traits [33–36].

After scrotal insulation, semen collection, cryopreservation, and computational image analysis; the aims of the present work were (1) to apply multivariate analyses to examine the set of alterations and interactions in sperm morphology and (2) to evaluate the importance of sperm parameters on the outcome of standard IVF and embryonic development.

2. Materials and methods

2.1. Scrotal insulation, semen collection, and cryopreservation protocol

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Federal University of Uberlândia (research protocol; #017-12). Three crossbred bulls (*Bos taurus* × *Bos indicus*), 30 to 36-month old, kept under grazing on an experimental farm and with previous fertility evaluation (≥70% morphologically normal sperm, ≥60% progressively motile sperm, and ≤20% of maximum sperm abnormalities) were used [37,38]. The bulls were subjected to a 72-hour scrotal insulation period (i.e., the scrotum was wrapped with cotton layers and covered with a plastic bag).

Before beginning the semen collection, false mounts with active restraint were performed and two successive ejaculates were collected from each bull by means of an artificial vagina once weekly before (preinsulation = Day 0) and throughout 56 days (Days 7, 14, 21, 28, 35, 42, 49, and 56) of the experimental period. The semen (combined ejaculates) was diluted to 100×10^6 spermatozoa/mL in Tris-egg yolk extender containing 7% (v/v) glycerol at 37 °C and loaded into 0.25-mL French straws. Cooling was started by transferring straws into a programmable freezing machine that performed (TK 3000, Uberaba, Brazil) the cooling (−0.25 °C/min to 5 °C) and freezing rates (1 step: −15 °C/min to −80 °C; 2 step: −10 °C/min to −120 °C). Subsequently, the straws were plunged into liquid nitrogen (−196 °C).

2.2. Sperm smear preparation

Semen preparation for computational evaluation was performed as described previously [13]. Briefly, after semen collection, two smears from each ejaculate were fixed with ethanol acetic acid (3:1, v/v) for 1 minute followed by 70% ethanol for 3 minutes. The smears were hydrolyzed for 25 minutes in 4-N hydrochloric acid, washed in distilled water, and air dried. Then, a droplet of 0.025% toluidine blue in McIlvaine's buffer (LabChem Inc., Zelienople, PA, USA) was placed over each smear, which was immediately covered with a coverslip.

2.3. Computational image analysis

2.3.1. Chromatin decondensation and heterogeneity

Gray-level digital images of spermatozoa were obtained randomly using a microscope (×1000) coupled with an image analysis system (Leica Microsystems Inc., Buffalo Grove, IL, USA). Displayed images consisted of shades of gray that varied from black (value of zero, lowest intensity) to white (value of 255, highest intensity). Threshold-based image segmentation was used [39], and at least 100 sperm heads were isolated for each smear. In addition, the average intensities of the gray levels within the sperm heads in each image were estimated by means of algorithms developed in the Scilab mathematical environment (Scilab Enterprises, Versailles, France).

After segmentation, the sperm heads were analyzed to obtain an average pixel value that made up each head. Subsequently, the difference between the standard value of the smear and the average value of each head was determined. This difference was transformed into a percentage of the average pixel value for the standard heads and used as a quantitative indicator of sperm chromatin decondensation (CD) [40]. In addition, sperm chromatin heterogeneity (CH) was determined by the coefficient of variation of the gray-level intensity for each sperm head [13].

2.3.2. Sperm-head morphometric variables

All sperm morphometric variables are shown (Table 1) and were determined using algorithms developed in the Scilab environment.

2.4. In vitro production of bovine embryos

Oocytes (n = 8,633) collected from slaughterhouse ovaries by follicular aspiration were subjected to maturation, fertilization, and *in vitro* culture. Briefly, cumulus oocytes complexes (COCs) were aspirated from follicles between 3 and 8 mm in diameter. Then, COCs with a homogeneous cytoplasm and several cell layers were selected and subjected to IVM in modified TCM 199 (0.2-mM pyruvate, 25-mM sodium bicarbonate, 75 μg/mL gentamicin, 1 μg/mL 17-β estradiol, 0.5-μg/mL FSH, and 100-UI/mL hCG) supplemented with 10% fetal calf serum (v/v). On average, 20 selected oocytes matured in drops (100 μL) over 22–24 hours at 38.5 °C in 5% CO_2 in air.

After IVM, the COCs were rinsed in Tyrode's albumin lactate pyruvate (TALP) medium and fertilized into drops (100 μL) of TALP supplemented with penicillamine (21.1 mM), hypotaurine (10.4 mM), epinephrine (1 mM), heparin (10 mg/mL), and (BSA; 6 mg/mL). For fertilization, frozen semen was thawed (37 °C for 1 minute), and sperm cells were obtained after treatment with a Percoll (45 and 90% gradient). The final sperm concentration added per fertilization drop was 1×10^6 sperm/mL. The sperm cells were coincubated with oocytes (19–22 hours) under the same conditions described for maturation.

After IVF, presumptive zygotes were denuded by repeated pipetting and washing, and then transferred to a culture dish (4-well) with modified synthetic oviduct fluid (SOFaaci; [41]) containing 5% fetal calf serum (v/v). Culture