Journal of Equine Veterinary Science 36 (2016) 10–14

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

journal homepage: www.j-evs.com

Original Research

Reevaluating the Sperm Nuclear Chromatin Decondensation Test by Sodium Thioglycolate of Stallions Spermatozoa

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ARTICLE INFO

Article history:
Received 8 June 2015
Received in revised form
18 September 2015
Accepted 12 October 2015
Available online 23 October 2015

Keywords:
Stallion
Sperm nucleus
Chromatin compaction
Sodium thioglycolate

ABSTRACT

The nucleus of mammalian spermatozoa is mainly composed of chromatin associated with protamines: highly basic proteins (around 7 kDa). These highly basic proteins, due to their cysteine content, can participate in the generation of disulfide bond. These characteristics permit typical condensed nuclear structure in mature spermatozoa, where the DNA becomes organized in compacted units, similar to nucleosomes, but with 60 kb of DNA. This shape is ultimately assumed in the epididymal maturation, and the level of compaction is closely related to epididymal function. In the present work, we present a modified method to evaluate sperm decompaction using sodium thioglycolate (ST). Stallion sperm were exposed to different ST concentrations and were embedded in agarose as a supportive medium. With the use of agarose, it was easier to identify patterns of decompaction with ST, and, thus, the use of a permeabilizing solution was not necessary. This was due to the utilization of ST to evaluate chromatin compaction of stallion sperm physically permeabilized and embedded in agarose matrix.

1. Introduction

In the seminiferous tubule of testis, where spermiogenesis occurs, spermatids nuclei undergo complex morphologic, biochemical, and physiological changes to complete their development. One of these changes is related to nuclei condensation due to the exchange of histones primarily by transition proteins, after highly basic arginine and cysteine rich protamines. Later, the nucleus will be composed of highly compacted chromatin during the epididymal transit because of the formation of disulphide bonds between free sulfhydryls on cysteine of protamines that will be oxidized to form disulphide bonds [1]. This produces protamine-DNA chromatin, generating tightly packed toroidal-like structures, containing up to 60 kb of DNA, in which the transcription and repair activities are inactivated and also related to morphology of sperm head and maturation state of sperm during epididymal transit [2].

As one of the features that can provide functional information of the sperm, several approaches for assessing the presence of disulfide bonds have been tested involving reductant agents such as including dithiothreitol (DTT) + sodium dodecyl sulfate (SDS) detergent, and sodium thioglycolate (ST) among others [3,4]; being their effect in the sperm nucleus mainly evaluated as changes in sperm head size.

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http://dx.doi.org/10.1016/j.jevs.2015.10.002

Sodium thioglycolate was chosen because, as was published by Fornés and Bustos-Obregón [5], the fact that it could freely pass the plasma membrane. In stallion sperm, ST tests have been used to study compaction level [6]. However, their observation and evaluation is not easy, because mainly to a weak and nonspecific staining, heterogeneous response of sperm to decondensation process, that evidenced it with several patterns of staining (color and size of halo). Moreover, to date, there is no evidence that the sodium thioglycolate across freely and without resistance through the plasma membrane of the sperm. If not the case, part of then response to decondensation depends on if the sodium thioglycolate effectively was incorporated to citosol and later reacting with sperm chromatin, aspect that was evaluated in this work.

Here, we propose the use of a new protocol of ST as a reducing agent for disulphide bonds, overcoming the lack of the effect in sperm with plasma membrane integrity, whereas with the original protocol, only sperm with their membrane disrupted presented reactive characteristics. This differs from the work of Fornés and Bustos-Obregón [5], in which all cells are expected to react with ST, and owing to the fact that the conditions in which the sperm cells are immobilized and attached to the slide for observation are different, being the agarose a supportive medium for controlled and homogeneous sperm sample decompaction.

2. Materials and Methods

2.1. Sample Preparation and Selection

In this study, only frozen semen samples were used. The samples were obtained from ejaculates of five stallions, with fertility probed with frozen semen, and with ages between 7 and 16 years old, from Heavy Draft breeds, property of Haras Militar Pupunahue (a member of the Fondef D08I1076 project), which explicitly approved the use of the samples for scientific research purposes.

Qualified samples were cryopreserved according to standard procedure, using a BotuCrio freezing extender. The ejaculates were collected using an artificial vagina, filtered to remove gel, diluted 1:1 in prewarmed skimmed milk (isothermal condition), and centrifuged at 1,000*g* for 20 minutes. Postcentrifugation sperm pellets were suspended in extenders previously tempered at 20°C, packed at 50×10^6 sperm/mL in straws of 0.5 mL, and cooled to 5°C for 90 minutes. They were subsequently exposed to liquid nitrogen vapors for 20 minutes at 4 cm over the liquid nitrogen level and finally submerged and stored in liquid nitrogen until analysis.

The cryopreservation samples were thawed at 37°C for 30 seconds before their evaluation.

In this study, only cryopreserved samples with a post-thawing sperm vitality evaluation over 40% were considered.

2.2. Sperm Vitality Evaluation

Vitality of the fresh sperm was evaluated using an eosin/nigrosin stain. A drop of the semen sample was mixed with a larger drop of eosin Y (2.5%), and nigrosin (5%) stains on a prewarmed slide with an applicator stick and a thin smear was made with another slide. After air-drying, the smear was observed (×40) with an LED illuminated view microscope (Nikon E200) for unstained heads of the spermatozoa (live) and stained or partially stained heads of the spermatozoa (nonviable). A total of 200 spermatozoa were counted to determine the percentage of live and nonviable spermatozoa. The average of three observations was considered a single data point.

In the laboratory, both cooled and thawed sperm samples were assessed using acridine orange (AO)/propidium iodide (PI) double staining. Sperm samples were mixed (1:1) on a tempered microscope slide with a staining solution AO/PI (20 and 10 μM, respectively) and were immediately analyzed using the viability module from the CASA System (Sperm Class Analyzer; Microptic, Spain) and observed at ×10 with the aforementioned LED illuminated view microscope (Nikon E200). During this procedure, viable and dead spermatozoa were observed with differential fluorescent emission in the head spermatozoa (green [525 nm] for viable and red [620 nm] for nonviable spermatozoa). Viability percentages were established from a count of at least 500 spermatozoa in each sample.

2.3. Sodium Thioglycolate Treatment

2.3.1. Fornés and Bustos-Obregón (protocol A)

A volume of 1 mL of 0.4-M ST pH 9 was added to 1 mL of 1×10^4 sperm suspension and incubated at room temperature for 15 minutes. The reaction was stopped using a 10-fold dilution with cold phosphate buffer (0.05 M, pH 7.2), centrifuged at 760*g* for 5 minutes, and the pellet was then resuspended in phosphate-buffered saline [5].

2.3.2. Modified Protocol (B)

A number of 1×10^4 sperm in 80 μL of low melting point (LMP) agarose 1% was mixed at 37°C (volume of 2-μL spermatozoa in 80-μL agarose) and placed in 40 μL over a 1% agarose LE glass slide prewarmed at 37°C, covered with coverslip, and left to cool for a maximum of 5 minutes at 4°C. The plasma membrane integrity was evaluated in parallel set of slides by AO/PI double staining in epifluorescent microscope.

In the not stained (AO/PI) set of slides, a drop of ST was added at different concentrations and incubated for 15 minutes (for the dose response experiment at a concentration of 0.2 mM until 200 mM) or 30 minutes (for standardized protocol, concentration 0.5 M). After incubation, the slide was dehydrated in ethanol (95% and 100%, 5-minutes each), air dried, and stained with Wright eosin methylene blue stain (Merck). The samples were stained until a layer of oxidized stain was observed (between 10 and 30 minutes), then washed with tap water, air dried, and mounted on a hydrophobic mountain medium. Resume of protocols are shown in Supplementary Fig. 1. For control of reductant agent, sperm cells were incubated in DTT, 2-Mercaptoethanol (BME), and ST at 0.5, 0.05, and 0.005 M diluted in distilled water, following the protocol just indicated. This is showed in Supplementary Fig. 2.