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Theriogenology
Theriogenology 66 (2006) 354–365
www.journals.elsevierhealth.com/periodicals/the

Nuclear status of immature and mature stallion spermatozoa

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Received 25 August 2005; accepted 16 October 2005

Abstract

'The highly packed chromatin of mature spermatozoa results from replacement of somatic-like histones by highly basic arginine- and cysteine-rich protamines during spermatogenesis, with additional conformational changes in chromatin structure during epididymal transit. The objective of the present study was to compare the nuclear characteristics of immature and mature epididymal stallion spermatozoa, using a variety of experimental approaches. Resistance to in vitro decondensation of chromatin, following exposure to SDS-DTT and alkaline thioglycolate, increased significantly in mature spermatozoa. Evaluation of the thiol-disulfide status (monobromobimane labeling) demonstrated that immature cells obtained from *ductulli efferentes* contained mostly thiol groups, whereas these groups were oxidized in mature cells collected from the cauda epididymidis. Based on atomic absorption spectrophotometry, maturation of stallion spermatozoa was accompanied by a 60% reduction in the Zn^{2+} content of sperm cells, concomitant with increased concentrations of this ion in epididymal fluid. Furthermore, the degree of disulfide bonding was inversely correlated with susceptibility of chromatin to acid denaturation (SCSA). Collectively, these data were consistent with the hypothesis that maturation of stallion spermatozoa involves oxidation of sulphydryl groups to form intra- and intermolecular disulfide links between adjacent protamines, with loss of zinc as an integral feature. These changes endow mechanical and chemical resistance to the nucleus, ensuring efficient transmission of the paternal genome at fertilization.
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Keywords: Sperm maturation; Chromatin decondensation; $Zinc^{2+}$; Thiol-disulfide status; Stallion

1. Introduction

During spermiogenesis, the nucleus of spermatids undergoes complex morphological, biochemical and physiological alterations. Their shape, size and condensation state change dramatically, due to exchange of histones by transition proteins and highly basic arginine- and cysteine-rich protamines [1,2]. These changes in the complex DNA-proteins eliminate the nucleosomal organization of chromatin, resulting in a tightly packed toroidal-like structure, containing up to 60 kb of DNA, in which the transcription and repair activities are inactivated [3,4]. The primary factor that induces compaction is thought to be protamine binding; it neutralizes the negative charge on the phosphodiester backbone of DNA [5]. In vitro studies of the kinetics of DNA condensation and decondensation induced by protamines and synthetic peptides have shown that the number of clustered arginine residues present in the DNA binding domain is the most important factor affecting the condensation and stability of the DNA-protamine complex, prior to the formation of inter-protamine disulfide cross-links [4]. Although the exact structure of the complex has not been determined, spectroscopic studies suggested that protamine binds to DNA in an extended conformation, with a footprint that covers −11 bp [5]. Protamine binding experiments, conducted in the presence of Zn^{2+}, suggested that a zinc finger would

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0093-691X/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2005.10.024

facilitate the binding of P2 protamine to DNA [6]. Although zinc-induced conformational changes in protamines have not been demonstrated, their presence appears to be important for sperm chromatin function.

In the epididymis, the gross morphology of the spermatozoon does not change, but biophysical and biochemical changes affect sperm architecture and composition; these changes include progressive formation of disulfide bonds that covalently link adjacent protamine chains [7]. Other sperm structures, e.g. outer dense fibers of the flagellum, also become stabilized by S–S linkages during epididymal transit. Thiol oxidation is also involved in the functional competence of the outer dense fibers, thereby affecting sperm motility [8].

There is abundant evidence regarding the structural organization of chromatin and its association with gene regulation [9]; chromatin condensation during sperm formation and its subsequent decondensation in the oocyte are essential for a successful transmission of the male genome. A serine/threonine protein kinase in mouse spermatids that seems to be essential for DNA condensation and male fertility was recently reported [10]. In the last decades, particularly since the development of in vitro fertilization techniques, nuclear status has been used for assessment of potential male fertility. However, there is still uncertainty regarding the fine architecture of the nucleus, the mechanism of disulfide bond formation, and the influence of Zn^{2+} in sperm physiology. Furthermore, species-specific differences in the chromatin-packing pattern, its susceptibility to reducing agents, and its zinc content, have been reported [11]. The objective of this study was to analyze the nuclear status of immature and mature epididymal stallion spermatozoa, utilizing decondensation tests, ultrastructural study of condensed and decondensed cells, sperm chromatin structure assays (SCSA), determination of thiol-disulfide status, and Zn^{2+} relative content.

2. Materials and methods

2.1. Chemicals

Unless otherwise indicated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Sample collection

Twenty healthy, thoroughbred stallions, 3–5 years of age, were surgically castrated. The epididymides were dissected from the testes; the proximal caput, corpus and cauda regions were separated, incised and squeezed to liberate their luminal contents. Spermatozoa were initially collected in a 0.9% NaCl solution, centrifuged ($700 \times g$, 10 min) and then washed three times in 0.9% NaCl by centrifugation ($700 \times g$, 5 min) before processing for each assay.

2.3. Sperm chromatin stability

Chromatin stability was tested by in vitro assays of decondensation induced by: (a) an anionic detergent, 0.5% sodium dodecyl sulfate (SDS; Calbiochem-Nova Biochem Co., San Diego, CA, USA) with 2 mM dithiothreitol (DTT; Bio-Rad (Richmon, CA, USA), in 0.05 M borate buffer, pH 9 [12]; (b) 0.4 M sodium thioglycolate, pH 9 [12]; and (c) 0.5% SDS in 6 mM EDTA [13]. Incubation times were 8, 10 and 30 min for SDS-DTT and 8, 10 and 15 min for alkaline thioglycolate (at room temperature), whereas incubation times were 15, 30 and 60 min (at 37 °C) for SDS-EDTA. The reactions were stopped by the addition of a fixative solution. Control (without treatment) and treated sperm cells obtained from caput, corpus and cauda epididymides regions were fixed in 4% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M phosphate buffer, washed twice in PBS, and processed for electron microscopy. For light microscopy, samples were fixed in 3:1 methanol/acetic acid (10 min) and stained with Giemsa, or directly observed by phase contrast microscopy (Zeiss-Axioplan, Oberkochen, Germany). Morphology was assessed by one observer, using pre-established criteria (form, size and light diffraction), as follows: (a) Group I or stable, with ordinary light diffraction or fully retained staining properties, no observable changes in area and configuration of sperm head (head length 6.5–7 μm); (b) Group II, with moderately swollen spermatozoa (head length >7.0 to <8 μm), partially refringent or lightly stained; and (c) Group III, with grossly swollen head (head length ≥ 8 μm), decreased light diffraction or further reduction of nuclear stain. Sperm head measurements were made with "Soft Imaging System Analysis™", with a Zeiss Axioplan video microscope; the percentages of stable, partially and decondensed spermatozoa were calculated. At least 200 sperm cells were evaluated and classified per each epididymal region and experimental condition. The effects of each decondensation treatment on spermatozoa obtained from proximal caput, corpus and cauda epididymides regions were tested by analysis of variance (one way ANOVA) and comparison of means by Tukey HSD test. Analysis of variance and comparison of means were also carried out between stable spermatozoa after SDS-DTT, SDS-EDTA and alkaline thioglycolate.

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