Theriogenology xxx (2014) 1–7

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Dense spermatozoa in stallion ejaculates contain lower concentrations of mRNAs encoding the sperm specific calcium channel 1, ornithine decarboxylase antizyme 3, aromatase, and estrogen receptor alpha than less dense spermatozoa

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ARTICLE INFO

Article history:
Received 11 November 2013
Received in revised form 18 March 2014
Accepted 16 April 2014

Keywords:
Sperm purification
Density gradient centrifugation
Gene expression
Spermatogenesis
Semen analysis

ABSTRACT

Stallions are unique among livestock in that, like men, they commonly receive medical treatment for subfertility. In both species, about 15% of individuals have normal semen parameters but are subfertile, indicating a need for novel analyses of spermatozoa function. One procedure for improving fertilizing capability of stallions and men is isolation of dense spermatozoa from an ejaculate for use in artificial insemination. In the current study, dense and less dense spermatozoa were purified by density gradient centrifugation from individual ejaculates from seven reproductively normal adult stallions. The RNA isolated from the spermatozoa seemed to be naturally fragmented to an average length of 250 bases, consistent with reports of spermatozoa RNA from other species. The DNAse treatment of RNA prepared from spermatozoa removed any genomic DNA contamination, as assessed by PCR with intron spanning primers for the protamine 1 (PRM1) gene. Concentrations of seven mRNAs in spermatozoa, correlated with the fertility of men and bulls, were quantified by reverse transcription polymerase chain reaction in dense and less dense spermatozoa. Concentrations of four mRNAs were two- to four-fold lower in dense spermatozoa compared with less dense spermatozoa: Encoding the spermatozoa-specific calcium channel ($P < 0.03$), ornithine decarboxylase antizyme 3 ($P < 0.02$), aromatase ($P < 0.02$), and estrogen receptor alpha ($P < 0.08$). In contrast, concentrations of three other mRNAs, encoding PRM1 and heat shock proteins HSPA8 and DNAJC4, were not different ($P > 0.1$). These results identify new differences in mRNA concentrations in populations of spermatozoa with dissimilar densities.

1. Introduction

Horses are a unique livestock species that share subfertility as a common medical problem with humans [1]. Clinical assessments of the reproductive potential of men and stallions are limited, as is the ability to predict their fertility. Evaluation of semen includes assessment of spermatozoa number, morphology, and motility and, to a lesser extent, assessment of spermatozoa quality with an assortment of fluorescent probes for various proteins. Blood concentrations of reproductive hormones (including testosterone, estrogens, luteinizing hormone, follicle-stimulating hormone, and inhibin) are of limited

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http://dx.doi.org/10.1016/j.theriogenology.2014.04.016

usefulness because of variability depending on the season, time of day, illness, medications, and environmental stressors. Both semen analyses and serum hormonal values can be misleading, that is, apparently normal for a subfertile individual or abnormal for a fertile one [2]. The best assessment of stallion fertility is pregnancy rate per estrous cycle in a large number of mares. However, this is costly in time and money [3]. New, laboratory-based tests with good predictive value for fertility would complement the available semen analyses.

Procedures have been developed for isolating dense spermatozoa from an ejaculate by centrifugation through colloid media. This was initially performed in fertility clinics for human semen in the middle 1980s [4], but is now becoming more widely used in assisted reproductive technologies for livestock species [5,6]. The purification of dense spermatozoa from semen improves spermatozoa motility, morphology, and chromatin integrity parameters compared with less dense spermatozoa and with unfractionated semen in human [4,7], equine [3,8,9], bovine [10,11], and porcine [12] species. The dense spermatozoa also have superior fertilizing capability *in vivo* [8,13–15] and *in vitro* [4,10–12,16] across those species. In human and livestock species, purification of dense spermatozoa may be performed for subfertile individuals before artificial insemination [8,13]. In livestock species, it can also be used on fertile males to increase the number of breeding doses from an ejaculate or to purify spermatozoa from viral pathogens [5]. Interestingly, such spermatozoa purification is considered biomimetic—mimicking the successful spermatozoon's change in environment in the female reproductive tract after mating [5].

Spermatozoa are very specialized cells that transport paternal genes to oocytes. Therefore, it was surprising when several thousand different mRNAs were recently identified in spermatozoa from different mammals, including bulls and men [17,18]. They include the mRNAs encoding protamine 1 (PRM1), protamine 2 (PRM2) and other proteins important to spermatozoa [19]. Many mRNAs in spermatozoa are synthesized immediately after meiosis and translated in elongating spermatids [20,21]. It seems that RNAs in spermatozoa are fragmented by an unknown mechanism, which may be important to stop translation late in spermatogenesis [22–24]. There is evidence that PRM1 and PRM2 mRNAs, which are very small, are delivered by spermatozoa to zygotes [25]. If other mRNAs in spermatozoa are fragmented and not functional as templates for protein translation, they are at least remnants of gene expression during late spermatogenesis [26]. Several studies of human spermatozoa mRNAs indicate that specific mRNAs could be useful for assessment for male fertility [26–28]. Intriguingly, dense spermatozoa from men and bulls have different concentrations of some RNAs than less dense or unseparated spermatozoa [7,17].

The objective of the current study was to compare quantities of selected mRNAs in dense and less dense spermatozoa purified from stallion ejaculates. The PRM1, PRM2, heat shock proteins HSPA8 and DNAJC4, ornithine decarboxylase 3 (OAZ3) and aromatase (CYP19A1) mRNAs were chosen from reports that identified their differential concentrations in spermatozoa from fertile and subfertile men [29–31]. The CATSPER1 and ESR1 mRNAs were also selected for quantification because knockout mice or men with natural mutations have impaired spermatogenesis and subfertility [32,33]. The results presented herein for the stallion are compared with similar relative quantification of mRNAs in dense and less dense sperm from men and bulls [7,17].

2. Materials and methods

2.1. Ejaculate collection and spermatozoa purification

All animal care and procedures were approved by the Texas A&M University Institutional Animal Care and Use Committee. Semen was collected two or three times from each of seven performance-bred American Quarter Horse stallions. An artificial vagina was used while the stallion mounted either a dummy or an estrogenized "teaser" mare. Semen parameters measured were volume of gel and gel-free fractions, spermatozoa concentration, morphology, and motility (% motility, % progressive motility, linear velocity, % straightness), as described previously [8]. Briefly, neat semen from each ejaculate was diluted 1:99 in buffered formal saline fixative and 100 spermatozoa were evaluated for morphology using differential interference contrast microscopy. The motility was measured using a Hamilton-Thorn motility analyzer [34].

Dense spermatozoa were purified from less dense spermatozoa and any epithelial cells by centrifugation. For each ejaculate, four 15-mL centrifuge tubes were prepared with 2 mL of 40% silica particle gradient media (EquiPure; Nidacon International AB, Mölndal, Sweden) on top of 80% gradient media [8]. One milliliter of neat semen was placed on top of each gradient. After centrifugation at 200× *g* for 30 minutes at room temperature, the less dense spermatozoa were harvested from the 40%:80% interface and the dense spermatozoa from the bottom of the tubes. Somatic cells (primarily epithelial cells) remain at the top of the gradient [35,36]. The purified spermatozoa fractions were pooled from the four gradients, washed in phosphate buffered saline, pelleted, snap frozen in liquid nitrogen and stored at −80 °C.

2.2. RNA preparation from spermatozoa and quality analyses

Total cellular RNA was isolated from purified spermatozoa samples with TriPure reagent (Roche, Indianapolis, IN, USA). Treatment with RQ1 DNAse (Promega, Piscataway, NJ, USA) removed possible contaminating genomic DNA. RNA concentration was measured with a Nanodrop ND-2000 spectrophotometer. Capillary electrophoresis analyses with a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) indicated RNA integrity values around 2.6 and an average size of about 250 bases for the fragmented RNA in spermatozoa, consistent with reports of others [22,23,36].

2.3. Quantitative reverse transcription of spermatozoa RNA

For quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) to accurately measure mRNA