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Fatty acids and plasmalogens of the phospholipids of the sperm membranes and their relation with the post-thaw quality of stallion spermatozoa

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Abstract

Fatty acids and plasmalogens were extracted from the phospholipids of the plasma membrane of stallion spermatozoa, to determine their relation with sperm quality after freezing and thawing. Sperm quality was rated using a quality index that combined the results of the analysis of sperm motility and velocity (CASA analysis), membrane status and mitochondrial membrane potential (flow cytometry) post thaw. Receiving operating system (ROC) curves were used to evaluate the value of specific lipid components of the sperm membrane herein studied as forecast of potential freezeability. From all parameters studied the ratio of percentage of C16 plasmalogens related to total phospholipids was the one with the better diagnostic value. For potentially bad freezers, the significant area under the ROC-curve was 0.74, with 75% sensitivity and 79.9% specificity for a cut off value of 26.9. Also the percentage of plasmalogens respect to total phospholipids gave good diagnostic value for bad freezers. On the other hand, the percentage of C18 fatty aldehydes related to total phospholipids of the sperm membrane properly forecasted freezeability with an area under the ROC curve of 0.70 with 70% sensitivity and 62.5% specificity for a cut off value of 0.32.

Keywords: Stallion; Sperm; Plasmalogens; Fatty acids; Flow cytometry; Cryopreservation

1. Introduction

Sperm technology has become an area of increasing interest for the equine industry [1]. One of the major problems of the equine species is the large inter-individual variability in sperm quality. Such variability is often ascribed to the fact that most stallions have been selected by performance and phenotype, and not for sperm quality either directly or indirectly. The physiological and biochemical reasons behind this variability are being slowly disclosed [2], and recent attempts include the predictive value of several markers for successful freeezability of a given ejaculate [2–7]. In many species including horses, peroxidation of lipids of the plasma membrane (lipid peroxidation, LPO) has been claimed to be a major factor involved in sperm quality after thawing [5,8,9]. The particular susceptibility of the sperm plasma membrane to peroxidative

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damage is due to a high cellular content of polyunsaturated fatty acids as well as their deficiency in protective enzymes, consequence of spermatozoa loosing most of their cytoplasm during spermiogenesis. Long chain polyunsaturated fatty acids (PUFAs) have been detected in the sperm membrane of humans and other mammals [10]. These unsaturated fatty acids give the plasmalemma the fluidity that the spermatozoon requires to participate in the membrane fusion events associated with fertilization. However, these molecules are also vulnerable to the attack by reactive oxygen species (ROS). Such peroxidative damage would disrupt the plasmalemmal fusogenity and its ability to support key membrane-bound enzymes such as AT-Pases. Moreover, alterations in the fluidity of the sperm membrane could alter the activation of signal transduction pathways, critical for sperm function. The aim of the present study was to disclose to what extent the composition of fatty acids and plasmalogens of the membrane of ejaculated stallion spermatozoa relates to their ability to survive freezing and thawing procedures.

2. Material and methods

2.1. Semen collection and processing

Semen (four ejaculates per stallion) was obtained from 7 Andalusian stallions individually housed at the Veterinary Teaching Hospital of the University of Extremadura, Cáceres, Spain. The stallions were maintained according to institutional and European regulations, and were collected on a regular basis (two collections/week) during the 2008 breeding season. Ejaculates were collected using a Missouri model artificial vagina with an inline filter to separate the gel fraction, lubricated and pre-warmed to 45–50 °C. The collected ejaculate was immediately transported to the laboratory for evaluation and processing. Only ejaculates with at least 60% progressive motility and intact membranes were included in the study. The filtered ejaculate was extended 1:3 (v/v) with INRA 96 (IMV, L 'Aigle, France), and centrifuged at 600 g for 10 min. The resulting sperm pellet was re-extended in freezing medium (Ghent, Minitüb Ibérica, Spain) to a final concentration of 100×10^6 spermatozoa per mL. The spermatozoa were slowly cooled to 4 °C within 1 h, loaded in 0.5 mL plastic straws and frozen horizontally in racks placed 4 cm above the surface of LN_2 for 10 min, after which they were directly plunged in LN_2. After at least 4 weeks of storage, straws were thawed in a water bath at 37 °C for 30 sec for analysis.

2.2. Sperm motility

Sperm kinematics was assessed using a CASA system (ISAS® Proiser Valencia Spain). The analysis was based on the examination of 25 consecutive, digitized images obtained from a single field using an ×10 negative phase contrast objective and a warmed (37 °C) stage. Two straws per stallion and freezing operation were thawed. Semen was loaded in a 20 μm depth Leja chamber (Leja Amsterdam, the Netherlands). Images were taken with a time lapse of one second; the image capture speed therefore being one every 40 ms, the number of objects incorrectly identified as spermatozoa were minimized on the monitor by using the playback function. For this, all the non-sperm objects present in the screen were removed from the analysis. With respect to the setting parameters for the program, spermatozoa with a VAP < 10 μm/s were considered immotile, while spermatozoa with a velocity > 15 μm/s were considered motile. Spermatozoa deviating < 45 % from a straight line were designated linearly motile.

2.3. Evaluation of mitochondrial membrane potential ($\Delta\Psi m$)

The lipophilic cationic compound 5,5′, 6,6′–tetrachloro-1, 1′, 3,3′ tetraethylbenzymidazolyl carbocianyne iodine (JC-1, Molecular Probes Europe, Leiden, The Netherlands) has the unique ability to differentially label mitochondria with low and high membrane potential. In mitochondria with high membrane potential, JC-1 forms multimeric aggregates emitting in the high orange wavelength of 590 nm, when excited at 488 nm. At the same excitement wavelength (488 nm), however, JC-1 forms monomers within mitochondria with low membrane potential, emitting in the green wavelength (525–530 nm). For staining, a 3mM stock solution of JC-1 was prepared in DMSO. From each sperm suspension, 5×10^6 spermatozoa were placed in one mL of PBS and stained with 0.5 μL JC-1 stock solution. The samples were incubated at 38 °C in the dark for 40 min before flow cytometric analysis [3].

2.4. Assessment of subtle sperm membrane changes and viability

Early sperm membrane changes and viability were determined as described in Peña et al [11], with modifications for adaptation to the equine species [2]. In