Animal Reproduction Science xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Simultaneous evaluation of superoxide content and mitochondrial membrane potential in stallion semen samples provides additional information about sperm quality

A. Johannisson[a,*], M.I. Figueiredo[a,1], Z. Al-Kass[a,b], J.M. Morrell[a]

[a] Division of Reproduction, Department of Clinical Sciences, Swedish University of Agricultural Sciences (SLU), Box 7054, 75007 Uppsala, Sweden
[b] Department of Surgery and Theriogenology, College of Veterinary Medicine, University of Mosul, Mosul, Iraq

ARTICLE INFO

Keywords:
Stallion
Spermatozoa
Cytometry
Mitochondria
Motility

ABSTRACT

An improved fertility prediction for stallions is of importance for equine breeding. Here, we investigate the potential of a combined staining of stallion spermatozoa for superoxide and mitochondrial membrane potential (MMP) for this purpose. Semen samples were analysed immediately after arrival at the laboratory, as well as after 24 h. Superoxide was measured by MitoSOXRed, while MMP was measured with JC-1. Menadione was used to stimulate superoxide production. In addition, other parameters of sperm quality, namely motility, membrane integrity, chromatin integrity, sperm kinematics and Hoechst 33258 exclusion were measured and correlated to superoxide production and MMP. Both bivariate correlations between measured parameters as well as multivariate analysis were performed. Measured values in the superoxide/MMP assay did not correlate with other parameters. However, there was a strong negative correlation ($r = 0.96$ after 0 h, $r = 0.95$ after 24 h) between membrane integrity and chromatin integrity. Moderate positive correlations were found between motility parameters and membrane integrity, as well as moderate negative correlations between motility parameters and chromatin integrity. The multivariate analysis revealed that membrane integrity, chromatin integrity and motility contributed to the first principal component, while the second was influenced by superoxide/MMP parameters as well as sperm kinematics. Storage of samples for 24 h decreased motility, chromatin integrity and membrane integrity. In conclusion, combined measurement of superoxide and MMP provides additional information not obtained by other assays of sperm quality.

1. Introduction

Equine breeders would like to have more effective methods to analyse sperm quality in the hope of using only good-quality sperm doses for AI (Morrell et al., 2017) and thus increase pregnancy rates. The proportion of motile spermatozoa together with the number and morphology of spermatozoa in a sample is commonly used to evaluate semen quality (Varner et al., 2015). However, it is not highly correlated with the fertilizing capacity of semen samples (Graham, 1996) since motility is only one of many attributes that a spermatozoon must possess to fertilize an oocyte. A more in-depth analysis of motility can be obtained by measuring kinematics with computer-aided sperm analysis (CASA), although differences in type of counting chamber used can influence the results and act as an additional source of uncertainty (Hoogewijs et al., 2012). Measurement of sperm quality can also be analysed by metabolic activity of

* Corresponding author.
E-mail address: anders.johannisson@slu.se (A. Johannisson).
[1] Present address: CECA/ICETA — Animal Sciences Centre, University of Porto, Vairao, Portugal.

https://doi.org/10.1016/j.anireprosci.2018.03.030
Received 29 November 2017; Received in revised form 21 March 2018; Accepted 28 March 2018

the spermatozoa (Gibb et al., 2014) for example, by measuring mitochondrial membrane potential (MMP, (Ortega-Ferrusola et al., 2009a)). Reactive oxygen species (ROS) are produced by all metabolising cells, thus the potential fertility of the stallion could potentially be indicated by a combination of ROS content and MMP levels contained in semen samples.

Mitochondria generate a major part of the ATP required for sperm metabolism, membrane function and motility, together with anaerobic glycolysis in the cytoplasm (Peña et al., 2009). Stallion spermatozoa have a high ROS production compared with other species, since sperm ATP production comes mainly from OXPHOS (Gibb et al., 2014, Varner et al., 2015). However, in some studies (Luo et al., 2013; Gibb et al., 2014) significant correlations were found between oxidative stress parameters and a number of motility parameters, suggesting that the most fertile ejaculates were those exhibiting higher levels of ROS production. A possible explanation for the relationship between the generation of ROS and fertility might be that the most fertile sperm populations are those exhibiting the highest levels of oxidative phosphorylation (OXPHOS), with ROS as a by-product of intense mitochondrial activity (Gibb et al., 2014).

The imbalance between the generation and degradation of ROS may be defined as oxidative stress (Baumber et al., 2000; Hossain et al., 2011). Under physiological conditions, ROS in low levels appear to be important for normal sperm functioning (Aitken et al., 1997) but excessive ROS-formation can affect cell viability (Aitken, 1995; Baumber et al., 2000). Hydrogen peroxide (H_2O_2) and superoxide (O_2^-) produced by spermatozoa have a functional role in Ca^{2+} buffering (Costello et al., 2009), apoptosis (Ortega Ferrusola et al., 2010), cell death (Peña et al., 2015), capacitation control (Agarwal et al., 2014) and sperm-oocyte fusion (Baumber et al., 2000; Aitken, 1995). Superoxide is quantitatively the predominant free radical produced by biological systems (Hybertson et al., 2011). Superoxide is short-lived and cell-impermeant (Peña et al., 2016). In the presence of nitrogen oxide (NO), superoxide can form the reactant peroxynitrite. Nitrogen oxide is more reactive than superoxide, and is produced in significant amounts by stallion spermatozoa (Ortega Ferrusola et al., 2009b). Immature, morphologically abnormal spermatozoa and seminal leukocytes are the main sources of ROS in ejaculates (Gibb et al., 2014). For many sperm preparation methods associated with assisted reproduction technology (ART), seminal plasma is removed, decreasing the antioxidant protection for spermatozoa, rendering them susceptible to oxidative stress (Baumber et al., 2000). Lower sperm quality and mitochondrial dysfunction may result in more ROS production during storage of sperm doses (Nohl et al., 1996) resulting in a negative relationship between the percentage of ROS in the sample and the foaling rate (Johannisson et al., 2014).

The purpose of the present study was to investigate the relationship of a combined measurement of MMP and superoxide content to other sperm quality parameters, and to determine if this relationship changes during cold storage of semen samples.

2. Material and methods

2.1. Semen collection

Commercial semen doses were obtained from 8 fertile Warmblood stallions, 4–18 years old, kept on a commercial stud in Sweden. Semen was collected up to three times per week during the breeding season; three ejaculates from six stallions and two ejaculates from two stallions were obtained in March and April. The semen was collected using an artificial vagina, Missouri model, using a phantom as a mount. Gel was removed using an in-line filter.

2.2. Sperm analysis

2.2.1. Sperm concentration

The concentration of spermatozoa in raw semen was measured immediately after ejaculation using a Nucleocounter SP-100 (Chemometec, Allerød, Denmark). Subjective motility was assessed by stud personnel. Semen AI doses were prepared by adding warm (37 °C) semen extender without antibiotics (Equiplus) to a final concentration of 10^9 motile spermatozoa (the standard dose for cooled semen in Sweden). Antibiotics (benzyl penicillin and dihydrostreptomycin) were added to combat bacterial contamination. The extended semen was aspirated into 20-mL syringes. Immediately after collection, the extended semen doses were driven to the laboratory (1 h drive) in a Styrofoam box containing a cold pack; such packaging, maintains the temperature of semen doses at approximately 7 °C for 24 h when the ambient temperature is 20 °C (Malmgren, 1998). On arrival at the laboratory at SLU, the sperm concentration was again measured using the Nucleocounter SP-100 to establish the sperm concentration for staining the spermatozoa for flow cytometry. Following initial sperm analysis, the semen was placed in a refrigerator and the analyses were repeated after 24 h.

2.2.2. Computer-aided sperm analysis (CASA)

Motility analysis (CASA) was performed using a SpermVision (Minitüb, Tiefenbach, Germany), which was connected to an Olympus BX 51 microscope (Olympus, Japan), when the samples arrived and again after 24 h. Aliquots (6 μL) of sperm samples were placed on a warm glass slide covered with an 18 × 18-mm coverslip. Motility in eight fields (~1000 spermatozoa) was evaluated at 38 °C using the SpermVision software program. The cell identification area was set at 14–80 μm^2 and spermatozoa were classified as follows: (1) immotile spermatozoa were defined as those with an average change in the orientation of the head of less than 17°; and (2) local (i.e. non-progressive) motile spermatozoa were defined as those covering a straight line distance (DSL) < 6 μm or having a circular movement with a radius < 35 μm and DSL < 30 μm. The kinematics measured were total motility (Motile), progressively motile (PMotile), curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), straightness (STR), linearity (LIN), Wobble (WOB), amplitude of lateral head deviation (ALH) and beat cross frequency (BCF).