Animal Reproduction Science 140 (2013) 62–69

Contents lists available at SciVerse ScienceDirect

Animal Reproduction Science

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

Reactive oxygen species in stallion semen can be affected by season and colloid centrifugation

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ARTICLE INFO

Article history:
Received 27 February 2013
Received in revised form 4 May 2013
Accepted 12 May 2013
Available online 18 May 2013

Keywords:
ROS
Stallion semen
Single Layer Centrifugation
Superoxide
Hydrogen peroxide

ABSTRACT

There are anecdotal reports that equine fertility may decline towards the end of the breeding season. Previous studies have examined differences in sperm quality between the breeding season and non-breeding season but few studies have investigated the proportions of superoxide or peroxide containing spermatozoa at different times during the breeding season. The purpose of this study was to measure the content of these reactive oxygen species (ROS) at the beginning and end of the Swedish breeding season, using flow cytometric analysis of the fluorescence produced after staining with hydroethidium and dichlorodihydrofluorescein diacetate. In addition, the effects of a new method of selecting good quality spermatozoa by colloid centrifugation, known as Single Layer Centrifugation (SLC), on ROS-content were investigated. Superoxide production by stallion spermatozoa was found to be higher at the start than at the end of the breeding season in Sweden ($22 \pm 16\%$ versus $9 \pm 6\%$, $P < 0.05$), whereas sperm motility was lower (total motility $80 \pm 9\%$ versus $90 \pm 6\%$, $P < 0.01$; progressive motility $55 \pm 12\%$ versus $60 \pm 8\%$, $P < 0.05$, at the beginning and end of the breeding season respectively). The mean values of the other parameters of sperm quality measured did not differ with time within the breeding season although differences did occur for individual stallions. SLC was found to select motile spermatozoa that contained less superoxide ($16 \pm 14\%$ versus $23 \pm 18\%$, $P < 0.01$) and less peroxide (0.3 ± 0.8 versus $1 \pm 2\%$, $P < 0.01$) than uncentrifuged controls, although they were capable of producing ROS when stimulated with menadione. This reduced peroxide production may contribute to the enhanced sperm survival (retention of motility) seen in the SLC samples during storage.

1. Introduction

The equine breeding season is influenced by daylight hours, with the onset of the season being brought about by an increase in day length and, conversely, the end of the season being initiated by decreasing day length. Mares show oestrus cycles only during the breeding season with a cycle length of approximately 22 days (Aurich, 2005). Stallions do not show testicular degeneration during the non-breeding season, as seen in some species, and semen is frequently collected for freezing during the months when there is no demand for fresh semen for artificial insemination. During the breeding season semen may be collected as often as every day (e.g. in Germany) or every 2–3 days (e.g. in Sweden).

There are anecdotal reports that fertility declines towards the end of the equine breeding season. However,

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http://dx.doi.org/10.1016/j.anireprosci.2013.05.006

it is likely that mares not conceiving earlier in the season are over-represented at the end of the season and since it may be more difficult for these mares to conceive, a false impression of declining fertility may be obtained. Although various studies have compared sperm quality for cryopreservation in and outside the breeding season, for example Janett et al. (2003a,b), Gamboa et al. (2010), Wrench et al. (2010), as yet there are few studies on sperm quality in fresh (cooled) semen at different times within the breeding season. Heckenbichleer et al. (2011) investigated some parameters of sperm quality (motility, morphology and membrane integrity) in ejaculates from various semen collection centers within the European Union but other parameters of sperm quality, such as content of reactive oxygen species, were not included in their study. Morte et al. (2008) measured lipid and protein oxidation in stallion spermatozoa but only compared the breeding and non-breeding seasons.

Sperm quality is a multifaceted concept, comprising both sperm factors and the properties of seminal plasma. It is known from studies in other species that some aspects of sperm quality may be affected by climatic conditions such as temperature, for example morphology and motility, which may be associated with fertility (Malmgren, 1997; Card, 2005), but seminal plasma quality may also play a role. Seminal plasma quality may be affected by differences in feed quality at different times of the year (Morrell, 1991). Contri et al. (2011) detected an effect of dietary supplementation with antioxidants on sperm quality in stallion ejaculates; they proposed that such supplementation affects the ability of spermatozoa to withstand the effects of oxidative stress.

High levels of reactive oxygen species (ROS) may be detrimental to stallion sperm survival in storage (Aurich, 2005). Since ROS are produced as byproducts of metabolism, highly metabolizing spermatozoa would be expected to produce more ROS than those with a lower metabolic rate. Single Layer Centrifugation (SLC) has been shown to select highly motile spermatozoa which retain their motility for longer than uncentrifuged spermatozoa (Johannisson et al., 2009; Morrell et al., 2010a) but their ROS-content has not been measured previously. The objectives of the present study were: (i) to determine whether sperm quality in commercial semen doses changes between the beginning and the end of the breeding season; and (ii) to measure ROS-content in SLC-selected and control (uncentrifuged) cooled stallion spermatozoa.

2. Materials and methods

2.1. Animals and husbandry

Semen was collected from 9 fertile warmblood stallions, 4–15 years old, at a commercial stud in Sweden (Flyinge AB), located at 55° 44′ N, 13° 21′ E. Semen was collected up to three times per week throughout the breeding season, which is standard practice on a commercial stud in this country. The stallions were allowed to mount a phantom and ejaculate into an artificial vagina (Colorado or Missouri, depending on the stallion), removing gel with an in-line filter.

A total of 34 semen doses were received at the start of the breeding season (four semen doses from each of seven stallions, three semen doses from two stallions) and 31 semen doses at the end of the breeding season (four from each of seven stallions, two semen doses from one stallion and one semen dose from the remaining stallion).

2.2. Sperm analyses

2.2.1. Sperm concentration

The concentration of spermatozoa in raw semen was measured immediately after ejaculation using a Spermacue photometer (Minitube, Munich, Germany). Warm (37 °C) semen extender (INRA96; IMV, L'Aigle, France) was added subsequently to provide AI doses of one billion motile spermatozoa, as is standard practice in Sweden. Syringes (20 mL) were filled with the extended semen before being placed in a Styrofoam box containing a cold pack, for overnight transport to the laboratory at the Swedish University of Agricultural Sciences (SLU). At SLU, the sperm concentration was measured using the Nucleocounter SP-100 (ChemoMetic, Allerød, Denmark) (Morrell et al., 2010b) for use when staining spermatozoa for flow cytometry.

2.2.2. Computer Assisted Sperm Analysis (CASA) for experiment 1

CASA was performed using a SpermVision (Minitüb, Tiefenbach, Germany), connected to an Olympus BX 51 microscope (Olympus, Tokyo, Japan) with a heated stage (38 °C). Aliquots (6 μL) of the sperm samples were pipetted on to a warm glass slide and an 18 mm × 18 mm cover slip was placed on top. Sperm motility of approximately 1000 spermatozoa in 8 fields of view was analyzed using the SpermVision software program using previously established settings (Aurich et al., 2007; Schäfer-Somi and Aurich, 2007). The cell identification area was set at 14–80 μm^2 with the following classifications: spermatozoa having an average orientation change of head of less than 17° were defined as immotile, those covering a straight line distance (DSL) less than 6 μm or having a circular movement with a radius less than 35 μm and DSL less than 30 μm were defined as having local (i.e. non-progressive) motility. For the purposes of this experiment, only the total and progressive motility were recorded.

2.2.3. Sperm morphology

An evaluation of sperm morphology was made using air-dried slides prepared from fresh sperm samples (500 spermatozoa) and also from aliquots fixed in formol-saline (200 spermatozoa) (Morrell et al., 2008). The proportion of morphologically normal spermatozoa was calculated by deducting the % abnormal spermatozoa from 100. Spermatozoa with distal cytoplasmic droplets were counted as normal.

2.2.4. Sperm Chromatin Structure Assay

Aliquots (50 μL) of the sperm samples were mixed with an equal volume of Tris–sodium chloride–ethylenediamine tetraacetic acid (TNE) buffer (0.15 M NaCl, 0.01 M Tris–HCl, 1 mM EDTA (ethylenediaminetetra-acetic acid), pH 7.4),