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Effect of sperm concentration on characteristics of frozen-thawed semen in donkeys

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ABSTRACT

In this study, the effect of donkey sperm concentration in the straw during cryopreservation on the quality of thawed semen was evaluated. Samples from seven adult Martina Franca jackasses were collected three times using a Missouri artificial vagina. After estimation of volume and concentration, raw semen was evaluated for motility using a computer-assisted sperm analyzer (CASA); viability and acrosome integrity were also determined. Fresh semen was then centrifuged and re-suspended at five different concentrations (100, 250, 500, 750, and 1000×10^6 sperm/ml) with a commercial extender, packaged in 0.5 ml straws, and frozen. After thawing, motility parameters, viability, and acrosome integrity were analyzed. The analysis of the data showed similar parameters of fresh semen compared with those of centrifuged and cooled samples. The sperm concentration in the straw affected the semen parameters analyzed after thawing, as suggested by evidence that when the concentration increased, the quality of the post-thawed semen decreased. Furthermore, the differences in total and progressive motility among samples at different concentrations are due to the immobilization of spermatozoa, as suggested by the finding that the percentage of static spermatozoa increased when the concentration increased. The reason for the impairment of semen quality when the sperm concentration increased was discussed. A great variability in cryo-resistance was found between jackasses but not within the same male, suggesting the presence of donkey males with semen that has acceptable and unacceptable freezing qualities.

1. Introduction

FAO regards several donkey breeds as endangered because of their small population size and serious risks of inbreeding (Vidament et al., 2009). Cryopreservation of germplasm is considered as the first option for the *ex situ* management of diversity in most endangered species (Wildt et al., 1995). Most of the procedures used for cryopreservation of donkey semen have been derived from those reported in horses. However, the results obtained in donkey semen cryopreservation were variable (Piao and Wang, 1988; Trimeche et al., 1996, 1998; Silva et al., 1997; Oliveira et al., 2006; Flores et al., 2008).

Horse semen cryopreservation requires many steps (extension, centrifugation, cooling, freezing, thawing, etc.), which interactively affect success. Stallion spermatozoa have been frozen at several cooling rates and in different packages (0.5-, 1-, and 4-ml straws; Martin et al., 1979; Cochran et al., 1983; Loomis et al., 1983; Love et al., 1989), plastic bags (Ellery et al., 1971), pellets (Pace and Sullivan, 1975), and aluminum tubes (Love et al., 1989). In addition, the number of spermatozoa per unit of volume varied greatly ($150–2000 \times 10^6$ cells/ml). The amount of spermatozoa per straw could be an important variable in horses and donkeys, in which several straws were thawed to obtain a usable inseminate dose. The increase in the sperm concentration during packaging

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allows a reduction in costs related to the disposable material and extender and reduces the space required for semen storage. Both these advantages are relevant, especially during the development of a sperm bank for the endangered species. Furthermore, deep artificial insemination using a large number/dose of sperm seemed to increase the fertility results (Oliveira et al., 2006). Despite these interesting advantages, few studies have specifically verified how sperm concentration at freezing could affect the post-thawing semen quality in mammals (Pena and Linde-Forsberg, 2000). In horses, this matter has been minimally explored, and the effect of the sperm concentration during packaging on the post-thawing quality of semen has been studied exclusively in terms of fertility (Heitland et al., 1996; Leipold et al., 1998; Nascimento et al., 2008). However, fertility is the result of several variables related to both the male and the female; thus, this specific parameters could be affected by variables other than the sperm concentration in the straw.

In the present study, the post-thawing sperm quality of donkey semen frozen at different concentrations (100, 250, 500, 750, and 1000 $\times$ 10^6 sperm/ml) was characterized. The quality of frozen-thawed semen was studied using a CASA system considering several in vitro parameters, such as viability, acrosome integrity, and kinetic evaluation.

2. Materials and methods

2.1. Animals

In the present study, semen was collected from seven Martina Franca jackasses (2–6 years of age, 350–420 kg in weight) with normal fertility and routinely used in artificial insemination. The jackasses were housed in box stalls with an open paddock, under natural light conditions, at the Veterinary Teaching Farm of the University of Teramo (Italy). The animals were fed with a standard diet composed of mixed hay (9 kg) and concentrate (2 kg) (Petrini First Equi Complet, Bastia Umbra, Perugia, Italy) twice daily, and water was freely available.

2.2. Semen collection

Semen was collected using a Missouri artificial vagina in the presence of a jenny in natural estrus. For each jackass, semen was collected three times. To reduce the variability due to the time of last ejaculation, double semen collection was performed with a 1-h interval, and the second ejaculate was used for this study. The semen collection was performed from April to June.

2.3. Semen evaluation

After collection, semen was filtered through gauze to remove the gel fraction. Total volume and gel-free volume were measured using a sterilized and graduated glass tube. Total sperm concentration was estimated using a Bürker counting chamber (Merck, Leuven, Belgium).

Kinetic evaluation was performed using the computer-assisted sperm analyzer (CASA) system IVOS 12.3 (Hamilton Thorne Biosciences, Beverly, MA, USA) for objective evaluation of motility as previously described (Contri et al., 2010a). For CASA analysis, samples were diluted at 30 $\times$ 10^6 sperm/mL using PBS; fresh and centrifuged samples were incubated at 37 °C for 5 min, and cooled and frozen-thawed semen samples were maintained at 37 °C for 10 min before analysis. Thus, a 2 μL aliquot of diluted semen was loaded into a 10 μm Makler chamber (Sefi Medical Instruments, Haifa, Israel), and 12 fields analyzed at random were considered. The settings used were those previously described in donkey semen (Contri et al., 2010b, 2012; Gloria et al., 2011). In the present study, 60 frames per second (Hz) and 30 frames per field were used. The donkey spermatozoa detection was verified using the playback function (Contri et al., 2010b). The motility parameters considered included total motility (TM; %), progressive motility (PM; %), average path velocity (VAP; μm/s), straight line velocity (VSL; μm/s), curvilinear velocity (VCL; μm/s), amplitude of lateral head displacement (ALH; μm), beat cross frequency (BCF; Hz), straightness (STR, as VSL/VAP; %), and linearity (LIN, as VSL/VCL; %). On the basis of their VAP, spermatozoa were classified as rapid (VAP > 75 μm/s), medium (75 μm/s > VAP > 25 μm/s), slow (VAP < 25 μm/s), and static cells (VAP = 0). Spermatozoa with VAP $\geq$75 μm/s and STR $\geq$80% were considered to be progressive cells.

Sperm membrane integrity was used to assess sperm viability, which was evaluated by propidium iodide (PI) and SYBR-14 fluorescent staining (Live/Dead sperm viability kit®, Molecular Probes Inc., Eugene, OR, USA) as previously described, with some formerly specified modifications in donkeys (Contri et al., 2010b). Briefly, an aliquot (200 μl) of diluted semen was incubated with 2.4 μM of PI and 20 nM of SYBR-14 at 37 °C under lightproof conditions. After 10 min, spermatozoa were fixed with 1 μl of 3% glutaraldehyde, and 6 μl of this solution was placed on a slide. A coverslip was applied, and the stained spermatozoa were examined under an Olympus BX51 epifluorescent microscope (Olympus Italy, Milan, Italy). Spermatozoa with bright green fluorescence (SYBR–14) were considered viable; those partially or totally red (PI) were considered dead.

Acrosome integrity was assessed using FITC-PSA dye (Sigma, St. Louis, MO, USA) as previously described for horse semen (Kavak et al., 2003). Briefly, 20 μM of FITC-labeled PSA solution in phosphate buffered saline (PBS) was prepared. FITC-PSA was added to a 300 μl aliquot of diluted semen. The stained sample was then incubated in the dark for 15 min at 37 °C. For each sample, a 10 μl drop per slide was prepared, a cover slip applied, and evaluated using an Olympus BX51 epifluorescent microscope. Sperm with a bright green fluorescent acrosomal region were considered as reacted. To determine viability and acrosome integrity, semen was diluted at 50 $\times$ 10^6 sperm/ml using INRA-Freeze, and the percentage was computed on at least 400 spermatozoa for each sample.

2.4. Experimental design

In the present study, INRA-Freeze extender (IMV Technologies, L'Aigle, France) was used in all the different steps of semen preparation and evaluation to avoid the possible effects of the chemical and osmotic properties of a