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Cryoprotective effect of glutamine, taurine, and proline on post-thaw semen quality and DNA integrity of donkey spermatozoa

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ABSTRACT

This study was conducted to evaluate the effect of amino acid addition to semen on post-thaw quality of donkey spermatozoa. Eighteen ejaculates were pooled and divided into aliquots which were cryopreserved in Gent A® containing 1% ethylene glycol (Gent-EG) and supplemented with 0 (as control), 20, 40, or 60 mM of glutamine, proline, or taurine. The greatest concentration (60 mM) of glutamine and taurine resulted in greater ($P < 0.001$) post-thaw sperm motility. Amino acid supplementation did not improve ($P > 0.05$) sperm morphology and membrane plasma integrity compared with the control samples. Whereas, improvement ($P < 0.05$) of acrosome integrity was observed with use of 60 mM glutamine. After thawing, there were no differences ($P > 0.05$) in the sperm DNA fragmentation index (sDFI) among treatments. The 60 mM glutamine and 40 mM taurine treatments, however, resulted in a reduction ($P < 0.05$) in sDFI values in the first 6 h of semen incubation, compared with the control samples. At 24 h, the sDFI values were less ($P < 0.05$) in all supplemented as compared with control samples, except for the 20 mM proline treatment group. In conclusion, supplementation of the Gent-EG extender with glutamine or taurine at 60 mM improved post-thaw donkey sperm quality. The addition of proline to the freezing extender, however, did not provide any significant enhancement in sperm quality, compared with the control group.

1. Introduction

Spanish donkey breeds (*Equus asinus*) are seriously at risk of extinction (Real Decreto 2129/2008, regulation of the National Catalogue of Endangered Species). There has been a rapid and substantial decrease in the population size and consequently in the genetic variability with these breeds (Aranguren-Mendez et al., 2001). At the present time, the Andalusian donkey is located mainly in Andalusia and Extremadura (southwestern Spain), and its population does not exceed 793 animals (DAD-IS FAO, 2014). The improvement of semen cryopreservation protocols for endangered donkey breeds would, therefore, allow the establishment of sperm banks and a more efficient use of cryopreserved donkey semen for insemination.

Cryopreservation induces irreversible damage to spermatozoa of horses mainly due to an osmotic imbalance at thawing (Morris et al., 2007), resulting in a significant loss of viable spermatozoa. A variable percentage of the surviving sperm population also has sub-lethal damage resulting in a reduced cell lifespan (Watson, 2000). Another source of osmotic stress is the permeating

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cryoprotectant used (Glazar et al., 2009), causing the cells to shrink or swell during the processes of cryoprotectant addition or removal, respectively. In recent years, studies have been conducted on donkey semen extenders, including cryoprotectants such as glycerol, ethylene glycol, dimethyl sulfoxide, dimethyl acetamide, and dimethyl formamide, to improve post-thaw quality of spermatozoa (Oliveira et al., 2006; Rota et al., 2012; Trimeche et al., 1998; Vidament et al., 2009). More recently, Acha et al. (2016) reported that the use of the semen extender, Gent® A (Minitüb GmbH, Tiefenbach, Germany), supplemented with 1% (v:v) ethylene glycol (Gent-EG) resulted in cryopreservation of Andalusian donkey sperm with greater viability outcomes. In addition, artificial insemination (AI) with cryopreserved donkey semen has been restricted in donkeys due to poor fertility rates (Rota et al., 2012). Indeed, the use of several reproductive strategies, including increasing in the number of spermatozoa per insemination dose, deep horn insemination or multiple inseminations, did not increase the fertility rate of donkey jennies (28.26%; Oliveira et al., 2016).

Sperm cryopreservation is also associated with overproduction of reactive oxygen species (ROS) that results in structural and biochemical alterations, such as lipid peroxidation (LPO) of sperm membranes, depletion of ATP, and DNA fragmentation, thereby reducing motility, viability and fertilizing capacity of spermatozoa (Aitken and Fisher, 1994; Bucak et al., 2007). Seminal plasma components may scavenge ROS to prevent oxidative-induced sperm damage (Baumber et al., 2000). Most protocols for donkey sperm cryopreservation, however, include the removal of seminal plasma. The addition of antioxidants (e.g., amino acids) to the freezing media can minimize the detrimental effect of ROS on sperm function and hence improve post-thaw semen quality and fertility with use of donkey spermatozoa.

Taurine is an aminoethanesulfonic acid which is found in high milimolar concentrations in spermatozoa and seminal plasma (Alvarez and Storey, 1983). The incorporation of taurine in the freezing extender has improved motility of frozen-thawed bull (Chen et al., 1993) and ram (Bucak et al., 2007) spermatozoa. Conversely, in dogs, Martins-Bessa et al., (2007) concluded that supplementation of the Uppsala extender with taurine (25–75 mM) does not improve sperm post-thaw mitochondrial activity or motility and viability.

Glutamine is an alpha-amino acid that is one of the components of the glutathione, which has an important role in protecting spermatozoa against ROS (Topraggaleh et al., 2014). The inclusion of glutamine (30–80 mM) in the freezing extender has, therefore, resulted in greater motility and velocity of stallion (Khlifaoui et al., 2005; Trimeche et al., 1999) and donkey (Trimeche et al., 1996) frozen-thawed spermatozoa. Phetudomsinsuk et al. (2009), however, reported that the inclusion of glutamine (50 mM) in freezing extenders resulted in a decrease in sperm motility and plasma membrane integrity of frozen-thawed stallion spermatozoa.

Proline is an alpha-amino acid that has also been detected at low concentrations in seminal plasma (Hinton, 1990). In horses, the addition of proline at 30–50 mM in modified INRA82 extender enhanced post-thaw sperm motility (Trimeche et al., 1999). The inconsistency in results obtained in these previous studies suggests a species-specific effect depending not only on the type of amino acids but also on the concentration of amino acids added to the extender (Cabrita et al., 2011). There have been no direct comparisons between the amino acids glutamine, proline, and taurine for donkey semen cryopreservation.

The aim of the present study was to compare the effect of Gent-EG supplementing with different concentrations (0, 20, 40, and 60 mM) of glutamine, proline, and taurine on post-thaw quality of Andalusian donkey sperm. Sperm quality was assessed by evaluating motility, morphology, plasma membrane integrity, sperm DNA fragmentation, and acrosome integrity.

2. Materials and methods

2.1. Experimental animals

This study was conducted at the Veterinary Teaching Hospital of the University of Cordoba (Spain). Eighteen ejaculates were collected from six healthy, mature Andalusian donkeys (6–15 years of age) of proven fertility. The jackasses were housed in individual paddocks and were fed daily with hay and grain, and water was freely available.

Semen was collected from the jackasses using a Missouri-model artificial vagina (Minitüb GmbH, Tiefenbach, Germany) with an in-line gel filter (Minitüb GmbH, Tiefenbach, Germany). A jenny in natural or prostaglandin-induced estrus (Luprostiol, 7.5 mg intramuscularly; Prosolvin, Intervet International B.V., Boxmeer, The Netherlands) was used to induce copulatory activity.

2.2. Semen evaluation

Immediately after collection the gel-free fraction of each ejaculate was evaluated to determine the volume, sperm concentration, and seminal pH. An aliquot of the ejaculate was also diluted in pre-warmed (37 °C) skim milk-glucose extender (EquiPro® A, Minitüb GmbH, Tiefenbach, Germany) without antibiotics to a final concentration of 25×10^6 spermatozoa/mL (Dorado et al., 2014). Aliquots of the diluted semen were subsequently used as needed to conduct the appropriate analyses.

Sperm motility was assessed using the computer-assisted sperm analyzer (CASA) system Sperm Class Analyzer (Microptic SL, Barcelona, Spain), as described by Miro et al. (2005) for donkey semen. Prior to the assessment of movement, aliquots of diluted semen were incubated at 37 °C for 5 (fresh semen) or 10 min (frozen-thawed samples). For each evaluation, three consecutive 5 µL drops of each diluted semen sample were evaluated using a phase contrast microscope (Eclipse 50i, Nikon, Tokyo, Japan) with a pre-warmed stage at 37 °C at 100× magnification. Two microscopic fields per drop were filmed randomly, including a minimum of 200 spermatozoa. Objects incorrectly identified as spermatozoa were minimized by using the playback function. Regarding the setting variables of the program, spermatozoa with a mean average path velocity (VAP) < 10 µm/s were considered immotile. Spermatozoa with VAP > 90 µm/s were considered as rapid, while spermatozoa deviating < 25% from a straight line were designated as linear motile. The measured variables of sperm motion were total motility (TM; %), progressive motility (PM; %), curvilinear velocity (VCL;