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Concentrations of non-permeable cryoprotectants and equilibration temperatures are key factors for stallion sperm vitrification success

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ABSTRACT

Vitrification is based on rapid freezing by direct exposure of sperm to liquid nitrogen (LN_2). This study evaluated the effect of non-permeable CPAs and equilibration temperature on stallion sperm quality after vitrification. In Experiment 1, different concentrations of sucrose (20, 50, 100 mM; mmol/L) and bovine serum albumin (BSA 1%, 5%, 10%) were compared including different temperatures for the equilibration (≈22 °C or 5 °C). Vitrification was performed dropping 30 µl sperm suspension directly into LN_2. In Experiment 2, conventional sperm freezing using 2.2% of glycerol in 0.5 ml straws, frozen in LN_2 vapours, was compared to the sucrose and BSA extenders (and its combination) producing the most desirable results. Sperm motility, plasma membrane and acrosome integrity were statistically compared between treatments. Vitrification after sperm cooling at 5 °C with sucrose 20 mM (S20) or BSA 1% (BSA1) resulted in the greatest values (mean ± SEM) for most of the sperm variables assessed. With use of the combination (S20 + BSA1/5 °C), there were greater values ($P < 0.001$) than freezing with glycerol for total (55.67 ± 2.99 vs 35.41 ± 2.96) and progressive sperm motility (38.32 ± 3.05 vs 14.42 ± 1.80), plasma membrane integrity (66.61 ± 2.69 vs 49.16 ± 2.60), intact-acrosomes (49.19 ± 2.60 vs 14.91 ± 1.57) and most of the kinetics assessed, respectively. In conclusion, stallion sperm can be vitrified after cooling at 5 °C using a combination of 20 mM sucrose and 1% BSA based extender and this is a promising alternative compared with conventional sperm freezing using glycerol.

1. Introduction

Cryopreservation of stallion sperm has been traditionally performed by slow freezing methods, including the use of different permeable cryoprotectants (CPAs); (Wu et al., 2015). With this technique, sperm are usually cooled (equilibration period) before freezing in nitrogen vapours or using a programmable biofreezer at slow rates (Clulow et al., 2008).

Vitrification is an alternative method of cryopreservation, based on rapid freezing, in which viable cells undergo glass-like solidification (Pradiee et al., 2015). This technology requires large concentrations of permeable CPAs (Isachenko et al., 2003), which increase the viscosity of the medium and prevent intracellular ice formation during cooling and warming, obtaining successful results

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in vitrification of oocytes and embryos (Rall and Fahy, 1985). Unfortunately, this technology failed when applied to vitrification of sperm due to the lack of tolerance of these cells to such concentration of CPAs (Macias Garcia et al., 2012; Oldenhof et al., 2017a). Recently, sperm vitrification was achieved by direct plunging of small volumes of sperm into liquid nitrogen without any permeable cryoprotectant (Isachenko et al., 2008). This technique has been termed `kinetic vitrification´ because ultra-rapid cooling rates prevents the intracellular ice formation and promotes the glass-like solidification of the sperm (Isachenko et al., 2004b); however, both vitrified and ice-forming regions may exist within the same extracellular solution and could be difficult to differentiate between vitrification and ultra-rapid freezing (Shaw and Jones, 2003). The `kinetic vitrification´ for sperm processing, therefore, means something different as compared with conventional term for vitrification associated with oocytes and embryos (Katkov et al., 2006), where both the intracellular milieu and the extracellular environment must become vitrified and there are inconsistent thoughts regarding the use of this terminology (Pradiee et al., 2015).

Kinetic vitrification has been successfully used for human and sperm of different animal species (Isachenko et al., 2004b, 2008; Sanchez et al., 2011; Merino et al., 2012; Pradiee et al., 2015). Tested vitrification media included the combination of carbohydrates and proteins (Hossain and Osuamkpe, 2007; Schulz et al., 2017) instead of permeable CPAs, mainly glycerol, traditionally used for slow freezing. It has been termed as `cryoprotectant-free vitrification´ in some reports (Isachenko et al., 2004a). A combination of sucrose and bovine serum albumin (BSA), as non-permeable agents, has previously been used in conventional freezing of stallion and donkey sperm as a strategy to avoid the toxicity of permeating CPAs (Diaz-Jimenez et al., 2018; Consuegra et al., 2018). Vitrification is also a simpler and cost-effective technique which makes it attractive for cryopreservation of sperm in commercial laboratories or even in field conditions (Pradiee et al., 2015). In recent reports, however, there has been little information about application of this technique, particularly in non-human species.

The aim of the present study was to evaluate the effect of non-permeable CPAs and temperature during the equilibration period on stallion sperm quality after vitrification and warming in comparison to conventional sperm freezing with glycerol.

2. Materials and methods

This study was approved by the Ethical Committee for Animal Experimentation of the University of Cordoba, in compliance with the Regional Government of Andalusia (project no. 31/08/2017/105) and the Spanish law for animal welfare and experimentation (RD 53/2013). All chemicals used were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise is stated. The base medium used for sperm processing and freezing as control was INRA96 (IMV Technologies, L'Aigle, France) adding different concentrations of bovine serum albumin (BSA) and sucrose (S) for sperm vitrification (see Experiment 1) or glycerol (GLY) for conventional slow freezing (see Experiment 2).

2.1. Semen collection and processing

Semen was collected from six clinically healthy stallions of different breeds (aged 6–15 years) using an artificial vagina in the presence of a mare in oestrus. Semen was collected from each animal twice a week on different sampling occasions obtaining fifteen ejaculates for Experiment 1 and eighteen for Experiment 2 ($n = 33$; 2–4 ejaculates per animal in each experiment). All the semen samples had at least a gel-free volume > 20 mL, sperm concentration > 146 × 10^6 sperm/mL, total sperm motility > 76.6% and progressive sperm motility > 49.9% evaluated as previously described (Ortiz et al., 2014). Fresh semen was diluted in a ratio 1:1 (v:v) with INRA96 and then aliquots were centrifuged 10 min at 600 × *g* (Alvarenga et al., 2012). The sperm pellets were re-extended to a final concentration of 50 × 10^6 sperm/mL in the control base medium (C, INRA96) adding BSA or S for sperm vitrification or GLY for slow freezing (see experimental design). After that, sperm samples were slowly frozen or vitrified as subsequently described.

2.2. Vitrification and warming of sperm

Sperm vitrification was conducted in spheres (small volumes) as previously described by Isachenko et al. (2008). A styrofoam box loaded with liquid nitrogen (LN_2) was used. Briefly, 30 µl droplets of the sperm suspension with the vitrification media (S or BSA) were plunged directly into LN_2 at distance of 10 cm from the surface. After contact with the LN_2 a sphere immediately forms and floats on the surface. It is important to avoid the placement of the spheres into a drop of greater volume. When solidification occurs (after about 24 s), the spheres descend to the bottom and can be easily collected by dissecting forceps. Spheres were packaged into 1.8 ml cryotubes maintained in LN_2 through the entire process (Arraztoa et al., 2017). Warming was performed after at least 24 h of storage in LN_2. Spheres were quickly submerged one by one (not more than five spheres) into 2 ml of INRA96 pre-warmed to 42 °C and gentle vortexing for 5 s. Post warm sperm suspension was centrifuged at 600 × *g*/10 min and the sperm pellet was resuspended with INRA96 to a final concentration of 25 × 10^6 sperm/ml for sperm evaluation.

2.3. Freezing and thawing

Semen samples were frozen following a standard protocol for stallions with modifications (Hidalgo et al., 2017). Briefly, diluted sperm pellets with the freezing medium with 2.2% glycerol (GLY) were slowly cooled to 5 °C within 2 h (Hidalgo et al., 2014) and then loaded in 0.5 ml plastic straws. The straws were frozen horizontally in racks placed 4 cm above the surface of liquid nitrogen (LN_2) for 10 min and placed into LN_2 tanks. After at least 24 h of storage in LN2, straws were thawed by immersion in a 37 °C water bath for 30 s, centrifuged (600 × *g*/10 min) and re-suspended with INRA96 for semen analysis.