Animal Reproduction Science 145 (2014) 123–129

Contents lists available at ScienceDirect

Animal Reproduction Science

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

Cholesterol-loaded-cyclodextrins improve the post-thaw quality of stallion sperm

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ARTICLE INFO

Article history:
Received 2 July 2013
Received in revised form 20 January 2014
Accepted 24 January 2014
Available online 6 February 2014

Keywords:
Equine
Sperm
Cholesterol
Reactive oxygen species
Membrane fluidity
Viability

ABSTRACT

An unacceptable proportion of stallion sperm do not survive the freeze-thaw process. The hypothesis of this study was that adding cholesterol to a stallion semen extender would stabilise the sperm membrane, resulting in an improved post-thaw semen quality in terms of increased sperm viability, membrane integrity and fluidity, and reduced oxidative stress. Semen was collected from three stallions and diluted in four extenders: TALP; TALP + 0.75 mg methyl-β-cyclodextrin–cholesterol (MβCD)/mL ($M\beta CD_{0.75}$); TALP + 1.5 mg MβCD-cholesterol/mL ($M\beta CD_{1.5}$); and Equipro. Following 15 min incubation, samples were centrifuged and diluted to 100×10^6 sperm/mL, frozen in 0.5 mL straws and stored in liquid nitrogen. Sperm from each treatment was assessed for progressive linear motility (PLM) and acceptable membrane integrity under hypotonic conditions on a phase contrast microscope at 1000× while viability, membrane fluidity and superoxide generation were assessed by flow cytometry. The $M\beta CD_{1.5}$ and $M\beta CD_{0.75}$ treatments had a greater proportion of viable sperm than the TALP treatment ($P < 0.01$). There was no effect of treatment on PLM or membrane integrity. The $M\beta CD_{1.5}$ treatment had a greater proportion of viable sperm positive for membrane fluidity than the TALP treatment ($P < 0.05$). The $M\beta CD_{1.5}$ and $M\beta CD_{0.75}$ treatments had a lesser proportion of viable sperm positive for superoxide generation than the TALP treatment ($P < 0.001$). This study has demonstrated that adding cholesterol to stallion sperm prior to cryopreservation increases post-thaw viability, with these viable sperm being of better quality in terms of increased membrane fluidity and reduced superoxide generation.

1. Introduction

Stallion semen does not survive the cryopreservation process as well as other species (most notably the bull) leading to significantly reduced conception rates following insemination with frozen-thawed semen compared to insemination with cooled semen (Sieme et al., 2003a). In addition, approximately one third of all stallions produce semen which is excessively damaged by the cryopreservation process and thus are categorised as 'poor freezers'. The standard semen cryopreservation process requires the diluted semen to be initially cooled to 5 °C and as the temperature decreases the sperm undergo a membrane lipid phase change, in which it transitions from a liquid to a gel phase. Peak phospholipid transition is thought to occur in stallion sperm at approximately 20 °C (Parks and Lynch, 1992) due to the loss of a hydrogen bound water molecule from the phospholipid head, increasing the van der Waals bonds between the lipid acyl chains, providing a tighter packing of the phospholipid bilayer (Crowe et al., 1990). This transition in stallion sperm has been associated with an increase in reactive oxygen species (ROS; Burnaugh et al., 2010), extracellular leakage of solutes (Drobnis et al., 1993), membrane destabilisation (Amann and Pickett, 1987) and even cell death.

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http://dx.doi.org/10.1016/j.anireprosci.2014.01.013

The process of freezing and thawing initiates an osmotic response in sperm which is highly dependent on the permeability and fluidity of the cell membrane to both water and cryoprotectants (Li et al., 2006). The membrane permeability of sperm to water is vital during the freeze-thaw process, as upon thawing the extracellular ice melts and cells are exposed to a hypotonic environment (Levin et al., 1976). The addition of a cryoprotectant can change the degree of water permeability exposing the cell to osmotic stress, hence, the composition of the freezing extender is essential for sperm survival (Hammerstedt et al., 1990; Sieme et al., 2003b). Cryoprotectants can permeate the sperm membrane causing shrinking and swelling of the sperm as a result of the addition and removal, respectively. Stallion sperm usually exhibit a lesser tolerance to osmotic stress in comparison with bull sperm (Ball and Vo, 2001), so upon thawing, the dilution of the cryoprotectant may incur an excessive intake of water leading to membrane damage or cell lyses (Guthrie et al., 2002). Therefore, the greater the osmotic resistance of stallion sperm both pre- and post-thaw, the greater the sperm quality and rate of cryosurvival.

The cholesterol/phospholipid ratio is thought to be a major influence on membrane fluidity during cryopreservation. Cyclodextrins are cyclic oligosaccharides of glucose containing a hydrophobic centre which can store lipids (Klein et al., 1995). Incubation with cholesterol loaded cyclodextrins prior to cryopreservation provides a greater percentage of viable (Pamornsakda et al., 2011), membrane intact (Moore et al., 2005) and both total and progressively motile stallion sperm, compared to a non-cholesterol control (Hartwig et al., 2013, Madison et al., 2013). The use of cyclodextrins can induce the addition (when they are preloaded with cholesterol) or removal of cholesterol from the plasma membrane of stallion sperm (Pamornsakda et al., 2011), thus altering its membrane fluidity and stability. Sperm from humans and rabbits have a high cholesterol/phospholipid ratio in comparison with stallions and do not experience excessive membrane damage due to a severe temperature decline during cryopreservation (Darin-Bennett and White, 1977). The addition of 1.5 mg of cholesterol loaded cyclodextrin to stallion sperm can increase this ratio from 0.36 to approximately 0.82 which is similar to values reported for human and rabbit sperm, resulting in reduced membrane damage during cooling due to a reduced liquid to gel phase transition temperature (Moore et al., 2005). Similarly, during thawing, the use of cyclodextrins to remove the membrane bound cholesterol increased the reverse phase transition temperature from 24 °C to 32 °C, again decreasing membrane damage (Oldenhof et al., 2012). For bulls (Purdy and Graham, 2004) and stallions (Hartwig et al., 2013), sperm treated with cholesterol loaded cyclodextrin retained a greater *in vivo* fertilising ability, in comparison with a non-cholesterol loaded control.

While the effects of cyclodextrin loaded cholesterol on the cryopreservation of stallion sperm has been previously investigated, it remains to be elucidated how the addition of cyclodextrin loaded cholesterol affects the post-thaw quality of stallion sperm, in terms of membrane fluidity and reactive oxygen species generation. The objective of this study was to assess the effect of the addition of methyl-β-cyclodextrin loaded cholesterol to stallion sperm on a range of *in vitro* variables, namely; viability, PLM, membrane integrity, membrane fluidity and superoxide generation.

2. Materials and methods

2.1. Preparation of methyl-β-cyclodextrin–cholesterol

Methyl-β-cyclodextrin (MβCD; Sigma, Ireland) was used to increase the solubility of non-polar cholesterol (Sigma). MβCD-cholesterol was prepared by firstly adding 1 g of MβCD to 2 mL of methanol, and 200 mg of cholesterol was added to 1 mL chloroform. The chloroform/cholesterol solution (450 μL) was then added to the MβCD/methanol solution and mixed using a vortex until a clear solution was obtained. The solvents were removed by nitrogen gas and the remaining crystals were stored at room temperature. Prior to use 50 mg of MβCD-cholesterol solution was added to 1 mL of tyrosine albumin lactate pyruvate media (TALP; NaCl 100 mM, KCl 10 mM, $MgCl_2$ 2 mM, sodium pyruvate 2.5 mM, lactate 20 mM, glucose 5.5 mM, HEPES 20 mM, $CaCl_2$ 2.1 mM, bovine serum albumin 6 mg/mL), and incubated in a water bath at 37 °C until use.

2.2. Experimental design

Semen from three Irish Sport Horse stallions of proven fertility, ranging between 6 and 15 years of age, was collected during the breeding season at a commercial stud using an artificial vagina. Two ejaculates were collected from each stallion with an interval of 3 days and on both collection days each ejaculate was processed separately, providing a total of six ejaculates. Following collection, the gel fraction was removed and volume and progressive linear motility (PLM) were assessed (as a quality control check; results not shown). Sperm concentration was assessed using a haemocytometer following which each ejaculate was split in four and diluted to 120×10^6 sperm/mL in one of four extenders, namely; TALP + 0 mg MβCD-cholesterol/mL of extender (TALP); TALP + 0.75 mg MβCD-cholesterol/mL of extender ($M\beta CD_{0.75}$); TALP + 1.5 mg MβCD-cholesterol/mL of extender ($M\beta CD_{1.5}$); and Equipro extender (Equipro; Minitüb, Germany). The samples were incubated for 15 min at room temperature to allow for incorporation of the MβCD-cholesterol before centrifugation at $500 \times g$ for 10 min at 32 °C (Oliveira et al., 2010). The supernatant was removed, sperm concentration was re-assessed and was diluted to 100×10^6 sperm/mL in Gent freezing extender containing 5% glycerol (Minitüb, Germany) in 50 mL centrifuge tubes. These tubes were placed on a laboratory roller and allowed to cool to 4 °C over 50 min (0.56 °C/min). Samples were then packaged into 0.5 mL straws (Minitüb, Germany), frozen to −110 °C over 20 min (5.7 °C/min) in a programmable freezer (Kryo 10 Series III, Planer Products Ltd., UK), followed by immersion and storage in liquid nitrogen until use.