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Substitution of egg yolk by a cyclodextrin-cholesterol complex allows a reduction of the glycerol concentration into the freezing medium of equine sperm

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ABSTRACT

The aim of this work was to completely replace the egg yolk a classical diluent for freezing equine semen by a cyclodextrin-cholesterol complex. At the same time, the reduction in the glycerol content used for cryopreservation and the incubation time between sperm and the freezing media were evaluated.

Horse ejaculates were frozen with four different freezing extenders: a frozen reference medium (IF) containing egg yolk and 2.5% glycerol and media without egg yolk but supplemented with 1.5 mg 2-hydroxypropyl-beta-cyclodextrin cholesterol (HPβCD-C) complex and containing either 1% (G1), 2% (G2) or 3% glycerol (G3). Three incubation times (90, 120 and 180 min) at 4 °C between the fresh semen and the different media were tested before freezing. Viability and motility analyses were performed with computer assisted semen analysis (CASA).

Results showed that the freezing media containing the HPβCD–C complex with 1%, 2% and 3% glycerol significantly improve the 3 in vitro parameters of post thawing semen quality (viability, progressive and total mobilities) compared to IF. The best improvement of the parameters was obtained with G1 medium and the longest contact time. The substitution of egg yolk by HPβCD–C complex allows the decrease of protein charge of the medium while favouring the cholesterol supply to membrane spermatozoa offering it a better resistance to osmotic imbalance and a better tolerance to the glycerol toxicity. Our results highlight that the egg yolk of an extender for the freezing of horse semen can be completely substituted by HPβCD–C complex.

1. Introduction

Despite a good quality of fresh stallion semen, about 20% of ejaculates do not tolerate freezing and do not reach the minimum quality criteria required after thawing [31]. These stallions having a poor freezability of their semen are called « poor freezers ». More and more studies are concerned by the research of an optimized freezing technique for stallion semen cryopreservation [29] but the reasons for poor sperm freezing in horses as in other species are not yet known.

Improvement of freezing techniques can be obtained by optimizing temperature curves and incubation times, but also the composition of the extender. The freezing extenders currently used are mainly composed of micellar milk proteins and egg yolk as source of phospholipids, and glycerol as cryoprotectant [26]. Glycerol remains the cryoprotectant conventionally used with a maximum proportion of 3.5% to obtain good results [32]. Beyond this value, glycerol becomes toxic for equine sperm. Some research groups replaced glycerol by protein-based agents or modified amino acids [2,27] and obtained encouraging results.

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The role of the freezing medium is critical to prevent premature capacitation of spermatozoa while preserving their energy potential, motility and fertilizing ability [6]. In this context, the preservation of the plasma membrane integrity of spermatozoa is essential during freezing and thawing. It is well known that spermatozoa membrane is sensitive to damages due to freezing in several species [5]. The damage of spermatozoa seemed not be due to the formation of intracellular ice crystals but rather to an osmotic imbalance during thawing [21].

Freezing can cause irreversible damage to sperm. Most of this damage occurs at the plasma membrane level when sperm cells pass from room temperature (22 °C) at a temperature of 1 °C. During this transition phase, the phospholipids are released from the plasma membrane resulting in an increase in membrane permeability, membrane rupture and then finally cell death [3]. Previous studies have shown that the cholesterol/phospholipids ratio of plasma membrane was a major determinant in the fluidity and stability of the plasma membrane during cryopreservation [9,19]. Cholesterol is a sterol lipid which plays a central role in many biochemical processes and is a major component of cell membranes that maintain their stability and integrity. To prevent rearrangement of phospholipids and to increase membrane fluidity at low temperatures, cholesterol can be added to the plasma membrane of different cell types, including sperm. Cholesterol can be easily embedded to plasma membrane using β-cyclodextrins [12].

β-cyclodextrins are cyclic heptasaccharides consisting of β (1–4) glucopyranose units. They are soluble in water but have a hydrophobic center that can transport cholesterol in or outside the plasma membrane [14]. Cholesterol can be easily incorporated into cyclodextrins. The addition of a cyclodextrin-cholesterol (CDC) complex in the bovine semen before cryopreservation showed higher percentages of sperm motility and more intact membranes after thawing in comparison to the untreated semen [28]. Similar results were also reported with equine sperm treated with a small amount of cyclodextrin-cholesterol complex [7]. A 2-hydroxypropyl-beta-cyclodextrin cholesterol (HPβCD-C) complex showed good results for the preservation of pig semen but has never been tested in horses [11].

The main objective of this study was to replace the egg yolk from a commercial extender by a stable and standardized (HPβCD-C) complex. During this study, we also tested several concentrations of glycerol since its presence appears toxic for horse semen and is considered as a factor affecting the sperm preservation in “poor freezer” stallions. Finally, the influence of the incubation time between sperm and the extender media was verified. Three parameters of post thawing semen quality were evaluated (viability, progressive and total motilities) and compared to the reference extender with containing egg yolk and 2.5% glycerol.

2. Material and methods

2.1. Animals

A draught horse, two sport stallions and a pony aged between 17 and 18 years old and collected at a frequency of three times a week were used. The stallions were known to produce frozen semen of acceptable quality (>30% of progressive motility) with the standard freezing technique. They were housed in individual boxes in the stables of European Horse Centre of Mont-le-Soie (Vielsalm, Belgium). Horses received hay and a commercial concentrate twice daily (morning and evening). The stallions were collected with a minimum interval of two days on a phantom with an artificial vagina type Colorado (IMV Technologies, L'Aigle, France). Five ejaculates of each stallion were used in the study.

2.2. Analysis of fresh semen

Once the ejaculate was collected, its volume was recorded. The sperm was kept in an incubator at a temperature of 37 °C and protected from light. The initial concentration of spermatozoa was determined using the NucleoCounter SP-100 solution with the detergent Chemometec (CHEMOMETEC, Allerod, Denmark). The concentration of dead sperm was also determined with this device for the viability of fresh semen following the manufacturer instructions.

Thereafter, a sample of fresh semen was diluted to a concentration of 20×10^6 total spermatozoa/mL in the commercial extender “INRA 96” (IMV Technologies, L'Aigle, France). The diluted sample was incubated for 5 min in the incubator 37 °C protected from light.

Total and progressive motilities were determined using 20 μL Leja cells in a computer assisted semen analysis (CASA) (IVOS version 12.0, Hamilton Thorne Research, Beverly, MA, USA). CASA settings were as follows: 37 °C, 60 frames per second (60 Hz), sperm size set between 4 and 6 pixels. The total mobility was defined when the Velocity Average Path (VAP) was greater than 15 microns per second and progressive motility was defined when the VAP was above 30 μm per second and straightness greater than 50% (STR). The analysis was repeated twice in five different fields (10 repetitions for each analysis and a minimum of 600 analyzed spermatozoa).

2.3. Preparation of cyclodextrin-cholesterol complex

The cyclodextrin-cholesterol complex (CDC) was prepared as described by Purdy and Graham [28] by replacing methyl-β-cyclodextrin by 2-hydroxypropyl-beta-cyclodextrin (HPβCD). Briefly, 1 g HPβCD (Sigma–Aldrich, Bornem, Belgium) was dissolved in 2 mL methanol in a glass tube. A 450 μL aliquot of cholesterol (Sigma–Aldrich, Bornem, Belgium) dissolved in chloroform (200 mg/mL) was added to the cyclodextrin solution and then mixed. The solvents were removed and the resulting crystals (powder) were kept in an Eppendorf tube at 22 °C until use.

2.4. Preparation of freezing extenders

Four freezing extenders were tested. The first (IF) is the freezing extender of reference used in our laboratory, the “INRA Freeze” (IMV Technologies, L'Aigle, France) that contains egg yolk and 2.5% glycerol. The other extenders were prepared with the medium based INRA 96 having the same composition than “INRA Freeze” but that does not contain egg yolk and glycerol. Thereby, the second extender (G1) is the medium INRA 96 containing 1.5 mg/mL of the 2-hydroxypropyl-beta-cyclodextrin cholesterol (HPβCD-C) complex and 1% of glycerol (VWR, Hasroode, Belgium) as cryoprotectant. The third extender (G2) is a mixture of INRA 96 containing 1.5 mg/mL of the HPβCD–C complex and 2% glycerol. The fourth extender (G3) is a mixture of INRA 96 containing 1.5 mg/mL of the HPβCD–C complex and 3% glycerol. These media were prepared in 2.5 mL tubes and then placed at 20 °C until use. Before use, they were warmed in a water bath at 37 °C.

2.5. Sperm freezing

Once the initial concentration of fresh semen was known, 4 Falcons of 15 mL containing 500×10^6 spermatozoa/mL (1/3 semen + 2/3 INRA 96) were prepared for each ejaculate. A cushion medium, consisting of a Iodixanol solution (Maxifreeze, IMV Technologies, L'Aigle, France) was placed in the bottom of the tube (0.5 mL) using a spinal needle before the centrifugation (1000 g,

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