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Optimization of the cryopreservation of dromedary camel semen: Cryoprotectants and their concentration and equilibration times

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ABSTRACT

Research into an optimal cryoprotectant, its concentration and equilibration time underlies the successful cryopreservation of dromedary camel spermatozoa. This study assessed the cryo-efficiency of different cryoprotectants, their concentration and equilibration time and any interactions. In experiment 1, semen samples (n = 4 males; 2 ejaculates/male) were frozen using Green Buffer containing one of four cryoprotectants (3% glycerol, ethylene glycol, methyl formamide, dimethyl sulfoxide) and using 4 equilibration times (10 min, 0.5, 1 and 2 h). Glycerol and ethylene glycol provided the best motility recovery rates and different equilibration times were not significant for any cryoprotectant nor were any interactions noted. However different equilibration times were pertinent for improved kinematic parameters BCF and VSL. In experiment 2, glycerol and ethylene glycol were evaluated at 4 concentrations (1.5, 3, 6, 9%) with 0.5 h equilibration (n = 4 males, 3 ejaculates/male). Sperm motility recoveries, kinematics and acrosome status were assessed. Higher values for LIN and STR were found with ethylene glycol. At 0 and 1 h post thaw 3 and 6% of either cryoprotectant resulted in better motility values than 1.5%. Acrosome integrity was compromised at 9% cryoprotectant. There were interactions between cryoprotectant and concentration in total motility at 0 and 1 h. For glycerol, total motility recoveries were best at 3–9%; for ethylene glycol 1.5–6% were best at 0 h and 3–6% at 1 h. In conclusion, 3–6% glycerol or ethylene glycol offered the best cryoprotection for camel sperm while different equilibration times were not critical.

1. Introduction

Camelid sperm are generally not tolerant to freezing and thawing procedures [44]. Therefore ongoing research to find optimal extenders and cryoprotectants is pertinent. The choice of a suitable cryoprotectant agent (CPA) and its concentration are well known to be essential issues that affect the outcome. However an optimal CPA, its concentration and equilibration time has not been established for the cryopreservation of dromedary camel sperm. Glycerol (Gly) is the most widely used cryoprotectant for the preservation of tissues and cells, including spermatozoa, with many studies indicating positive outcomes from its use [28,36,40]. The positive effects of this cryoprotectant may be related to its increasing viscosity upon cooling; such might inhibit ice crystal growth in the freezing extender which in turn would prevent rising salt concentrations that might cause osmotic distress and damage sperm membranes [8,24]. The inclusion of Gly in both dromedary and Bactrian camel sperm cryopreservation protocols has been reported [1,21,46]. However, Gly can also be cytotoxic causing osmotic stress due to its low permeability through the plasma membrane as a consequence of its high molecular weight [31]; therefore its use can also lead to problems in capacitation and the acrosome reaction [32].

Alternative CPAs have been studied in various species with the aim of improving sperm freezing procedures. Amides such as dimethyl formamide, dimethyl acetamide and methyl formamide (MF) have been used in freezing extenders for sperm from dogs [29], rams [45], horses [6] and pigs [11]. The cryoprotective effect of these amides is attributed to their low molecular weight, viscosity and hydrophilic behaviour. Compared to Gly amides can reduce osmotic stress [10] and the formation of intracellular ice crystals [11]. Crichton et al. [18] used MF for the successful cryopreservation of cholesterol-supplemented dromedary camel sperm.

Lovelock and Bishop [39] discovered the protective effect of dimethyl sulfoxide (Me2SO) on freezing damage to living cells. The

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penetration of Me2SO is rapid due to its lower molecular weight relative to Gly [11]. Any deleterious effects of Me2SO may be more toxic than osmotic [50]; it has been used for semen cryopreservation in the rabbit [33], bull [58], wolf [34] and carp [61]. In a study on dromedary camel sperm cryopreservation, Abd El-Salaam [1] found better results using Me2SO compared to Gly or the combination of both, Ethylene glycol (EG) has also been used to cryopreserve sperm with encouraging results [31,32]; however, while it has been used in cryopreservation studies of alpaca sperm [52], the literature does not record its use for the preservation of camel sperm.

Just as critical to optimal cryopreservation is the concentration of CPA used to preserve viability and reduce cell damage [14]. Species differ in the requirements of their sperm for different concentrations of CPAs with a range of 2–10% being generally applied to preserve mammalian spermatozoa. The reason for this variation among species relates to differences in the composition and functionality of the sperm membrane. Thus it is necessary to set up a specific concentration of CPA to successfully freeze sperm for any species. The optimum concentration may vary according to the nature of the CPA, other osmotically active components in the extender and the freezing procedure. Dromedary camel sperm have been frozen in 2.5% MF [18], 2–6% Gly [1,22] and 2–6% Me2SO [1].

Equilibration time also plays a major role in the success of cryopreservation. In studies on camel sperm, equilibration times vary from 0 min to 4 h [1,18,22].

Therefore the aim of the present study was to evaluate different CPAs (Gly, EG, MF and Me2SO) for the preservation of dromedary camel sperm as well as to determine appropriate concentrations and equilibration times for each of them in order to set a standard protocol.

2. Materials and methods

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich Co (St Louis, MO, USA). Green Buffer® (GB) was purchased from IMV (L'Aigle, France).

2.1. Animals and semen collection

Four dromedary camel males from the Camel Reproduction Centre (Dubai, UAE) were used in this study. Semen was collected between March and May using an artificial vagina [56]. Semen samples were immediately transported to the lab and placed in a 37 °C water bath. All animal procedures were carried out according to the guidelines of the Animal Care and Use Committee of the Camel Reproduction Centre, UAE.

2.2. Experimental extenders

The basic medium used for initial sperm extension was Tris-Citrate-Fructose Buffer (TCF, pH = 6.9) composed of 300 mM TRIS, 94.7 mM citric acid and 27.8 mM fructose [23]. Bovine serum albumin (0.05%), EDTA (10 mM) and 4% egg yolk were added and the solution was sterilized by filtration (0.22 μm).

Sperm freezing was performed in a two-step procedure. A mixture of 80% (v/v) of GB and 20% (v/v) egg-yolk served as fraction A (FA). Fraction B (FB) was FA with the addition of different CPAs (Gly, EG, MF, Me2SO) to result in final concentrations of 1.5, 3, 6 or 9%, depending on the experimental design.

2.3. Sperm cryopreservation

Briefly, extended semen (1:1: v/v in TCF) was incubated with Papain (P5306: 0.1 mg/ml) at 37 °C for 20–30 min to facilitate liquefaction. During this time the mixture was constantly gently pipetted to break down the gel. Papain inhibitor (E3132; E64) was added (10 μm/L) and semen incubated for an additional 5 min. After centrifugation at 800 × g for 10 min to remove the seminal plasma, the pellet was re-suspended in Fraction A to a final concentration of 200 million spermatozoa/mL and examined for motility. Only collections ≥50% motility were used for experiments. Samples were cooled in a water jacket to 5 °C (2 h) and then further diluted with Fraction B (1:1) to a final concentration of 1.5, 3, 6 or 9% of CPA. Sperm were then loaded into 0.5 mL straws. After an equilibration interval of 10 min, 0.5, 1 or 2 h depending on the experimental design, straws were placed 4 cm above the surface of liquid nitrogen for 15 min. Then straws were plunged into liquid nitrogen for storage.

Frozen semen samples were thawed at 65 °C for 10 s and assessed for motility and acrosome integrity.

2.4. Sperm assessment

Motility was measured by means of a computer-assisted semen analysis (CASA) system (CEROS II®; Hamilton Thorne; MA; USA). For each evaluation, a 3 μL sperm sample was placed in a disposable counting chamber (MicroToolTM, Cytonix, USA) and five fields were analysed. Total and progressive motility and kinematic parameters were recorded. The kinematic parameters were: average path velocity (VAP); straight line velocity (VSL); curvilinear velocity (VCL); amplitude of lateral head displacement (ALH); beat cross frequency (BCF); straightness (STR) and linearity (LIN).

Acrosome integrity was evaluated with eosin-nigrosin stain. A semen sample was diluted 1:1 with stain solution (5% eosin, 10% nigrosin in 0.1 M citrate solution), smeared on a glass slide and dried on a warm plate. A total of 100 spermatozoa were assessed per sample and the percentage of intact acrosomes was calculated, using the presence of an intact apical ridge as the criterion for an acrosome-intact spermatozoon.

2.5. Experimental design

The present research was performed in two experiments. In experiment 1, a completely randomized block design with a 4 × 4 factorial arrangement was conducted to test main effects of CPAs (Gly, EG, MF and Me2SO) and equilibration times (10 min, 0.5, 1 and 2 h) with 8 replications per experiment unit (n = 4 males; 2 ejaculates per male) on total and progressive motility recoveries and kinematic parameters evaluated before and after freezing (0 and 1 h). Based on results of experiment 1, in experiment 2 a 2 × 4 factorial arrangement was used to evaluate the effect of CPAs (Gly or EG) and CPA concentrations (1.5, 3, 6 and 9%) and a 0.5 h equilibration time on motility recoveries and kinematic parameters before and after freezing (0 and 1 h) and also acrosome integrity at 0 and 1 h post thaw, with 12 replications (n = 4 males; 3 ejaculates per male).

2.6. Statistical analysis

Effect of CPAs, equilibration times, concentrations and their interactions were analysed by generalized linear model (GLM), with means compared by Duncan's multiple range method. Total and progressive motility post-thaw were expressed as recoveries estimated marginal means ± SEM. Kinematic parameters and acrosome integrity were expressed as absolute estimated marginal means ± SEM. All analyses were performed using SPSS 11.0 for Windows. The level of significance was set at $P < 0.05$.