Animal Reproduction Science 163 (2015) 75–81

Contents lists available at ScienceDirect

Animal Reproduction Science

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

Cryopreservation of stallion spermatozoa using different cryoprotectants and combinations of cryoprotectants

Zhuangyuan Wu[a], Xinbiao Zheng[b], Yongming Luo[b], Fei Huo[b], Hong Dong[b], Guoting Zhang[b], Weihao Yu[c], Fang Tian[a], Liangjun He[a], Jingbo Chen[a,b,*]

[a] College of Animal Science and Technology, Shihezi University, Shihezi 832000, Xinjiang, China
[b] Institute of Animal Science, Xinjiang Academy of Animal Science, Urumqi 830000, Xinjiang, China
[c] The Breeding Center of Yili Horse, Zhaosu 835600, Xingjiang, China

ARTICLE INFO

Article history:
Received 30 May 2015
Received in revised form
26 September 2015
Accepted 30 September 2015
Available online 3 October 2015

Keywords:
Frozen semen
Equine spermatozoa
Horse
Cryoprotective agents
CPA

ABSTRACT

The present study investigates the effects of five cryoprotectants (CPAs) and cryoprotectant combinations on the post-thaw total motility, progressive motility, viability, mitochondrial membrane potential and acrosome integrity in stallion spermatozoa. In Experiment 1, the objective was to compare the impact of different concentrations (2.5%, 3.5% and 5%) of a single CPA, including glycerol (Gly), ethylene glycol (EG), dimethyl sulphoxide (DMSO), methyl formamide (MF), and dimethylformamide (DMF) for stallion spermatozoa cryopreservation. In Experiment 2, two or more CPAs were used to assess whether this improved post-thaw spermatozoa quality. Gly, MF and DMF, were used to prepare seven combinations of freezing extender with different mixtures of cryoprotectant, and the 3.5% Gly, MF and DMF were used as a control group. The results show that post-thaw total motility, progressive motility, viability, and mitochondrial membrane potential for all concentrations of EG and DMSO were less than the 3.5% and 5% Gly and MF and 2.5% and 3.5% DMF ($P < 0.05$). Use of the 3.5% concentration resulted in the greater post-thaw total motility and progressive motility than the 2.5% and 5% concentrations for all CPAs. The results for the use of different combinations of cryoprotectant indicate there are differences in progressive motility and viability. The viability with the use of Gly(2/3) + MF(1/3) was 44.65% and was greater than the Gly(1/3) + MF(1/3) + DMF(1/3) (30.96%), MF(2/3) + DMF(1/3) (35.05%), Gly (32.21%) and MF(33.76%) ($P < 0.05$). The progressive motility with the use of the MF(2/3) + Gly(1/3) combination was 36.0% and was greater than in the DMF (25.0%) and MF(2/3) + DMF(1/3) (22.7%) ($P < 0.05$). These results suggest that using the appropriate cryoprotectant combination instead of a single cryoprotectant can improve horse spermatozoa cryopreservation.

1. Introduction

In modern horse breeding, frozen semen is popular because of its advantages compared to cooled-shipped semen. The semen from a large part of the stallion population, however, cannot be used for semen freezing because of unsatisfactory post-thaw spermatozoa quality and fertility rates (Alvarenga et al., 2004; Vidament et al., 2002; Samper and Morris, 1998). Furthermore, large variations in semen quality after the freezing–thawing process and varying pregnancy rates after insemination occur between different stallions and ejaculates (Oehninger et al., 2000; Samper and Morris, 1998). Therefore, the

* Corresponding author at: College of Animal Science and Technology, Shihezi University, Shihezi 832000, Xinjiang, China.
E-mail address: chenjb126@126.com (J. Chen).

http://dx.doi.org/10.1016/j.anireprosci.2015.09.020
0378-4320/

continuing development of artificial insemination techniques with frozen-thawed spermatozoa in the equine industry requires the improved quality of frozen samples and the minimization of individual variability among stallions.

In 1949, Polge et al. reported as a result of the "chance" discovery of the cryoprotective function of Gly during efforts to preserve avian spermatozoa in the frozen state. This report identified key elements that have an important role in the field of bio-preservation, including the need for a CPA (Polge et al., 1949). Gly has become widely used as a CPA for the frozen spermatozoa of various animals. In particular, the use of frozen bull spermatozoa has been a great success. However, the use of Gly has not been as successful for the cryopreservation of stallion spermatozoa, and a great variability of success is observed between individual stallions (Guay et al., 1981). Based on experimental studies of stallion spermatozoa, the most important factors causing cryoinjury are the toxicity caused by unequal distribution of CPAs, such as Gly, and the osmotic stress caused by dehydration of the extender and the cells during freezing and again at thawing (Morris et al., 2007; Morris, 2006). The cryoprotective capacity of any cryoprotectant agent varies widely across cell and tissue types (Karow, 1969); therefore, the efficacy of each cryoprotectant agent must be studied for each cell type.

A large number of reports have been published regarding the freezing of ejaculated spermatozoa with several CPAs (Hoffmann et al., 2011; Nur et al., 2010; Medeiros et al., 2002; Morrier et al., 2002; Molinia et al., 1994). However, previous studies have provided inconsistent results. Neves et al. (1995) found that pregnancy rates were desirable with semen frozen using EG. Tracey (1999) evaluated five cryopotectants, Gly, EG, diethylene glycol, propylene glycol and DMSO at four molarities (0.5, 1.0, 1.5 and 2.0 mol/l) on multiple ejaculates from seven fertile breeding stallions. Overall, use of DMSO provided for the most desirable post-thaw motility and viability values and superior post thaw results for stallions that had semen for which use of Gly resulted in poor freezing capacity. Other studies used amides as cryoprotectants for stallions, with more significant improvements for semen from stallions with semen that could not be effectively frozen using Gly (Alvarenga et al., 2005). Some studies have revealed a significant improvement of the fertility of stallion semen frozen with DMF compared to Gly (Medeiros, 2003; Moffet et al., 2003). By contrast, Mantovani et al. (2002) report that use of EG reduced the percentage of motile and progressive motility for spermatozoa after thawing as compared with use of 3% Gly. Spermatozoa frozen using Gly as the cryoprotectant had greater percentages of motile and progressively motile spermatozoa compared to spermatozoa frozen using DMF (Moore et al., 2006). Use of MF and DMF protect stallion spermatozoa from cryodamage as effectively as Gly (Squires et al., 2004). Other experiments showed no differences in fertility rates for mares inseminated with semen that was frozen with extenders containing DMF or Gly. For example, daily inseminations of mares resulted in pregnancy rates of 46% and 50% for semen processed with freezing extenders containing 2% Gly or 2% DMF, respectively (Vidament et al., 2002).

The objective of the present study is to explore the effects of various CPAs and cryoprotectant combinations in the INRA96 extender on the plasma, acrosomal, mitochondrial membranes and motility of frozen-thawed stallion semen.

2. Materials and methods

2.1. Stallions and semen collection

A total of six adult Yili stallions with proven fertility were housed at The Breeding Center for Yili Horses, Xingjiang China, and four horses were used for Experiment 1 and five for Experiment 2. Semen was collected from these stallions in September on a regular basis (two collections each week) using an artificial vagina. Six ejaculates per stallion were processed. After collection, raw semen was filtered to remove the gel portion.

2.2. Semen freezing and thawing

Filtered semen of each ejaculate was diluted (1:1) in INRA96, consisting of a HGLL solution containing Hank's salts, glucose and lactose, buffered with HEPES and supplemented with a purified fraction of native phosphocaseinate (Batellier et al., 1997). Extended samples were cooled at 22 °C for 10 min and then centrifuged at $600 \times g$ for 10 min. After centrifugation, the pellet was re-suspended in INRA96 supplemented with 2.5% clarified egg yolk to obtain 200×10^6 spermatozoa cells/ml. Then, 15 ml of re-suspended semen was pooled from every stallion and the pooled sample was allocated to Experiments 1 or 2.

In Experiment 1, re-suspended semen was aliquoted into 15 samples. Each sample was diluted with INRA96 containing 2.5% clarified egg yolk and 5%, 7%, or 10% of one of five different CPAs (Gly, EG, DMSO, MF and DMF). This resulted in final concentrations of 100×10^6 spermatozoa/ml and 2.55, 3.55 and 5% CPA.

In Experiment 2, the semen was aliquoted into 10 batches and diluted with an equal amount of INRA96 containing 2.5% clarified egg yolk and two-fold the final concentration of desired additional cryoprotectant combinations (Table 1).

The semen was loaded into 0.5 ml polyvinyl chloride straws (IMV-Technologies, L'Aigle, France), sealed with a filling and sealing machine (IMV-Technologies, L'Aigle, France), and then cooled to 4 °C over 75 min. Freezing was performed with a programmable freezer (nitrogen freezer, automatic Mini-Digitcool, IMV-Technologies, L'Aigle, France) (−60 °C/min until −140 °C). The straws were stored in liquid nitrogen and then thawed in a water bath at 37 °C for 30 s immediately before semen analyses.

2.3. Assessment of motility

The motility parameters were evaluated using computer-assisted analyses (CEROSII, version 13, Hamilton Thorne, Beverly, MA, USA). For each analysis, 5 μl of diluted semen was mounted on a disposable Leja counting chamber (fixed height of 20 μm, Orange Medical, Brussels,