Journal of Equine Veterinary Science 40 (2016) 56–61

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

journal homepage: www.j-evs.com

Original Research

Effect of Multiple Freezing of Stallion Semen on Sperm Quality and Fertility

Jutta Sielhorst [a,b], Christiane Hagen [a,c], Daphne Behrendt [a,c], Barbara Schuette [b], Dominik Burger [d], Gunilla Martinsson [c], Harald Sieme [a,*]

[a] Clinic for Horses—Unit for Reproductive Medicine, University of Veterinary Medicine Hanover, Hanover, Germany
[b] Veterinary Clinic Domaene Karthaus, Duelmen, Germany
[c] Hanoverian National Stud Celle, Celle, Germany
[d] Swiss Institute of Equine Medicine, Agroscope and University of Berne, Avenches, Switzerland

ARTICLE INFO

Article history:
Received 8 December 2015
Received in revised form 29 January 2016
Accepted 31 January 2016
Available online 5 February 2016

Keywords:
Cryopreservation
Sperm
Multiple freezing
Stallion
Fertility

ABSTRACT

To increase the efficiency of equine semen, it could be useful to split the artificial insemination dose and refreeze the redundant spermatozoa. In experiment I, semen of 10 sires of the Hanoverian breed, with poor and good semen freezability, was collected by artificial vagina, centrifuged, extended in INRA82 at 400 × 10^6 sperm/mL, and automatically frozen. After this first routinely applied freezing program, semen from each stallion was thawed, resuspended in INRA82 at 40 × 10^6 sperm/mL, filled in 0.5-mL straws, and refrozen. These steps were repeated, and sperm concentration was adjusted to 20 × 10^6 sperm/mL after a third freezing cycle. Regardless of stallion freezability group, sperm motility and sperm membrane integrity (FITC/PNA-Syto-PI-stain) decreased stepwise after first, second, and third freezing (62.3% ± 9.35, 24.0% ± 15.4, 3.3% ± 4.34, $P \leq .05$; 29.6% ± 8.64, 14.9% ± 6.38, 8.3% ± 3.24, $P \leq .05$), whereas the percentage of acrosome-reacted cells increased (19.5% ± 7.59, 23.9% ± 8.51, 29.2% ± 6.58, $P \leq .05$). Sperm chromatin integrity was unaffected after multiple freeze/thaw cycles (DFI value: 18.6% ± 6.6, 17.2% ± 6.84, 17.1% ± 7.21, $P > .05$). In experiment II estrous, Hanoverian warmblood mares were inseminated with a total of 200 × 10^6 spermatozoa of two stallions with either good or poor semen freezability originating from the first, second, and third freeze/thaw cycle. First-cycle pregnancy rates were 4/10, 40%; 1/10, 10%; and 0/10, 0%. In conclusion, as expected, sperm viability of stallion spermatozoa significantly decreases as a consequence of multiple freezing. However, sperm chromatin integrity was not affected. Pregnancy rates after insemination of mares with refrozen semen are reduced.

1. Introduction

The use of frozen-thawed semen in equine practice is increasing in the last years [1–3]. Despite of individual and breed variation in cryosurvival and a more intense management, satisfactory pregnancy results increase the horse breeder's acceptance for frozen-thawed semen in the equine practice.

Cryopreserved sperm enables artificial insemination irrespective of the availability and location of the donor. Thus, long-lasting storage in liquid nitrogen allows worldwide breeding activities, parallel involvement in sport activities, and insemination with valuable ejaculates beyond the life time of the stallion [4].

New techniques and insemination protocols facilitate pregnancies with "low-dose" inseminations of less than 50 × 10^6 spermatozoa [5]. Cryopreserved semen may be

* Corresponding author at: Harald Sieme, Unit for Reproductive Medicine, University of Veterinary Medicine, Hanover, Germany.
E-mail address: harald.sieme@tiho-hannover.de (H. Sieme).

0737-0806/$ – see front matter © 2016 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jevs.2016.01.014

even more efficiently used via fertilization in vitro. Repeatable methods for in vitro fertilization (IVF) have not be established in the horse and are still in the focus of laboratory research [6,7], but efficient intracytoplasmatic sperm injection (ICSI) with high cleavage (70–80 %) and blastocyst formation rates (20–30 %) has recently paved the way of in vitro embryo production into clinical application [8]. The number of spermatozoa needed for injection is very small; therefore, it displays an alternative if a stallion is no longer able to produce viable spermatozoa and each straw of frozen semen is literally irreplaceable [9].

Spermatozoa previously cryopreserved from a limited, deceased, or currently infertile stallion is likely to have been frozen in commercially available 0.5-mL straws at a high concentration of 200–400 $\times$ 10^6 spermatozoa/mL. Thawing even a single straw could waste a high percentage of sperm for assisted reproductive techniques (ARTs) or low-dose insemination. It could be useful to split the AI dose, dilute, and refreeze the redundant spermatozoa in smaller aliquots. Furthermore, the feasibility of multiple freezing may reduce storage capacity and allow refreezing of accidentally short-term thawed semen. It opens up opportunities for sex determination. Previously, routinely frozen spermatozoa can be thawed and sex predetermined using flow cytometric sorting of spermatozoa at a specialized location which may be remote from donor males or female recipients and refrozen in a second cryopreservation step to produce offspring of a predetermined sex by low-dose insemination or ARTs.

In human medicine, first studies focusing on the resistance of spermatozoa to cryoinjury after repeated thaw-refreezing cycles [10–12] were performed to provide additional opportunities for conception in men who have banked a limited number of spermatozoa such as cancer patients. Verza et al [13] recovered motile spermatozoa after five and two refreeze-thawing cycles in normozoospermic and oligozoospermic men, respectively.

For different purposes, semen refreezing has been applied in various species including bulls [14–16], rams [17,18], rabbits [19], fish [20], and brown bear semen [21]. Limited data are available regarding the effects of multiple freezing and thawing cycles on equine sperm quality in the horse [9,22]. McCue et al [22] briefly demonstrated the potential feasibility of refreezing stallion spermatozoa, with refrozen samples attaining approximately 70% of initial postthaw motility, but did not use semen for AI. As far as we know, no study evaluated the ability of refrozen spermatozoa to produce pregnancies via conventional artificial insemination in the horse. The aim of the present study was to analyze sperm motility, membrane, acrosome, and chromatin integrity after multiple freeze/thaw cycles and evaluate pregnancy rates after inseminating mares with refrozen semen.

2. Materials and Methods

2.1. Experiment I

2.1.1. Animals

In experiment I, 10 sires of the Hanoverian breed between 5 and 21 years of age were divided into two groups according to their semen freezability preceding the experiment. “Good freezers” (n = 5) showed >50% progressively motile sperm (PMS) immediately after semen collection and ≥35% PMS postthawing, whereas “bad freezers” (n = 5) reached 10–35% PMS postthawing. Semen samples used for the studies were aliquots from routine semen collections performed for the commercial artificial insemination program of the stud. Semen collections were performed every other day using an artificial vagina and a breeding phantom (both model “Hannover”; Minitüb, Tiefenbach, Germany).

Stallions were kept in box stalls, bedded with straw, were fed oats and hay three times a day, and water was freely available. Stallions were held according to national regulations and institutional animal care and use protocols.

2.1.2. Semen Processing

Semen was collected by artificial vagina, diluted in modified milk extender INRA82 (100 $\times$ 10^6 spermatozoa/mL), centrifuged (10 minutes., 600*g*), and extended in INRA82 including 2% clarified egg yolk (diluted 1:1 in aqua bidest, centrifuged 10 minutes, 15000*g*) at 400 $\times$ 10^6 spermatozoa/mL. The final glycerol concentration was adjusted to 2.5% at +22°C. After 120 minutes slow cooling in a water beaker at 5°C, semen samples were automatically frozen in 0,5-mL straws (+5°C → −140°C; 60°C/min).

After this first routinely applied freezing program and storage for 24 hours (−196°C), semen from each stallion was thawed (30 seconds, 37°C), evaluated, resuspended in INRA82 with a final glycerol concentration of 2.5% to evaluate the influence of multiple freezing cycles at 40 $\times$ 10^6 sperm/mL, filled in 0.5-mL straws, and refrozen. These steps were repeated, and semen concentration was adjusted to 20 $\times$ 10^6 sperm/mL after a third freezing cycle.

2.1.3. Semen Evaluation

The motility, membrane, acrosome, and chromatin integrity of spermatozoa after the first, second, and third freeze/thaw cycle were assessed by computer-assisted motility analysis (CASA) and flow cytometry.

2.1.3.1. Sperm Motility. The percentage of PMS was assessed using the CASA-system MIKA Motion Analyser Windows version 1.1 (Stromberg. Mika, Montreux, Switzerland). After thawing (30 seconds; 37°C), the semen sample was diluted to 25 $\times$ 10^6 sperm/mL and incubated 2 minutes before analysis at 37°C. Five microliter sperm was filled in prewarmed markler chambers (Selfi-medical Instruments, Haifa, Israel) and analyzed with a digital camera that recognizes 32 frames/s. Five fields were evaluated per sample. Cells moving slower than 10 μm/s were considered immotile, whereas cells moving ≥25 μm/s were considered to be progressively motile.

2.1.3.2. Membrane and Acrosome Integrity. The percentages of plasmamembrane-intact (PMI; PI negative) and acrosome-reacted sperm (positive acrosomal status [PAS]; fluorescein isothiocyanate conjugated to peanut agglutinin [FITC-PNA] positive) were flow cytometrically estimated in the FITC/PNA-Syto-PI-assay. Semen samples were diluted with Tyrode’s media to 5 $\times$ 10^6 sperm/mL. After staining 500 μL extended semen sample with 2 μL SYTO 17 (0.5 mM;