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Effects of an extension of the equilibration period up to 96 hours on the characteristics of cryopreserved bull semen

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ABSTRACT

This study was designed to investigate the effects of an equilibration period up to 96 hours and three extenders (AndroMed, OPTIXcell, and Triladyl) on the quality of cryopreserved bull semen and to evaluate, whether an extension of the equilibration time to 72 hours does affect fertility in the field. One ejaculate of 17 bulls was collected and divided into three equal aliquots and diluted, respectively, with the three extenders. Each aliquot was again divided into five parts and equilibrated for 4, 24, 48, 72, and 96 hours before freezing in an automatic freezer. Sperm motility, plasma membrane and acrosome integrity (PMAI), and DNA fragmentation index (% DFI) were measured during equilibration. In addition to the parameters measured during equilibration, the percentage of viable sperm cells with high mitochondrial membrane potential (HMMP) was measured immediately after thawing, and after 3 hours of incubation at 37 °C. Sperm motility was assessed using CASA, and PMAI, HMMP, and % DFI were measured using flow cytometry. Equilibration time did affect all parameters before freezing ($P < 0.01$), and also the extender affected all parameters except HMMP ($P < 0.05$). After thawing, all parameters except HMMP immediately after thawing were influenced by the equilibration period ($P < 0.001$), whereas all parameters except % DFI immediately after thawing were influenced by the extender ($P < 0.001$). The changes of semen characteristics during 3 hours of incubation were also dependent on the equilibration time and the extender used in all parameters ($P < 0.01$). In the field study, semen of nine bulls was collected thrice weekly, processed using Triladyl egg yolk extender, and frozen in 0.25 mL straws with 15×10^6 spermatozoa per straw. In total, the nonreturn rates on Day 90 after insemination (NRR90) of 263,816 inseminations in two periods were evaluated. Whereas semen collected on Mondays and Wednesdays was equilibrated for 24 hours in both periods, semen collected on Fridays was equilibrated for 4 hours in period one and equilibrated for 72 hours in period 2. No differences in NRR90 could be found ($P > 0.05$). In conclusion, extension of the equilibration time from 4 hours to 24–72 hours can improve motility and viability of cryopreserved semen after thawing. The extent of improvement in semen quality is dependent on the extender used. Prolongation of the equilibration period from 4 hours to 72 hours had no effect on fertility in the field.

1. Introduction

During the development of bovine semen freezing technology, each step between semen collection and freezing has been carefully evaluated, including duration of

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equilibration. In a review, Pickett and Berndtson [1] deduce from a series of fertility trials that slow cooling and equilibration at 5 °C are important for optimal fertility and recommend an equilibration time of 4–18 hours. The equilibration time is thought to be important for sperm membranes to adapt to low temperatures [2,3] and to enable the translocation of water, hence decreasing the damage by ice nucleation during freezing-thawing [4].

There is no agreement on what time of equilibration is best for semen quality after cryopreservation but there is a desire to control this step to optimize the production line in commercial AI centers. In recent literature, there is a wide range of equilibration periods reported: no equilibration at all [3,5], 30 minutes [6], 1.5 to 4 hours [3,5,7–10], 18 to 28 hours [10–12]. In experiments where it was examined, whether an equilibration period is necessary at all, 2 hours [5], or 4 hours of equilibration [3] delivered better results than cryopreservation without equilibration. Therefore, the equilibration period seems to be necessary for good semen quality after cryopreservation. When an equilibration period of 3–4 hours was compared with an overnight equilibration (18 or 24 hours), there was a higher post-thaw motility with the longer equilibration period [10,11], although no difference in fertility could be found when comparing 4 and 28 hours of equilibration period [11].

Muiño et al. [2] evaluated extenders with and without egg yolk using a prolonged equilibration time of 18 hours and found higher sperm survival and longevity for the egg yolk–containing extender. However, there is few data in literature about the effect of different extenders, with and without egg yolk, using a prolonged equilibration period.

Currently, there is a trend against using animal products in extenders because of hygienic risks, the lack of quality standards, and the presence of steroid hormones, which may reduce the fertilizing capacity of spermatozoa [2]. Substances of animal origin represent a risk for microbial contamination with the subsequent production of endotoxins capable of damaging the fertilizing capacity of spermatozoa [13].

With the aim to optimize the production line of a commercial AI center and improve the quality of semen collected and frozen on Fridays, we examined, whether it is possible to prolong the equilibration time up to 96 hours, using extenders with and without animal products without compromising sperm quality and fertility.

2. Materials and methods

2.1. Semen collection and processing

A total of 17 bulls (Brown Swiss n = 7, Red Holstein n = 7, Limousin n = 2, and Holstein Friesian n = 1), aged between 18 and 36 months were used for the experiment. One ejaculate of each bull fulfilling minimum standards of progressive motility (70%) and sperm concentration (500×10^6/mL) was processed with three different extenders to obtain a final sperm concentration of 60×10^6 spermatozoa per mL. The extenders used were Triladyl (Minitube, Tiefenbach, Germany), a TRIS-egg yolk–based extender, and the two extenders AndroMed (Minitube, Tiefenbach, Germany) and OPTIXcell (imv, L'Aîgle, France) containing no animal originating substances. Each aliquot was once again divided into five parts and equilibrated at 4 °C for 4, 24, 48, 72, and 96 hours before packaging at 4 °C in 0.5 mL straws with a concentration of 60×10^6 spermatozoa per mL. Thereafter, the straws were frozen in an automatic freezer (Microdigitcool, imv, L'Aîgle, France) and stored in liquid nitrogen at −196 °C.

2.2. Semen laboratory analysis

Semen characteristics were assessed after equilibration times of 4, 24, 48, 72, and 96 hours as well as after freezing-thawing and pooling the contents of three straws immediately (0 hours) and after additional 3 hours of incubation at 37 °C.

2.2.1. Sperm motility assessment with CASA

The IVOS II CASA system driven by software version 14 (Hamilton Thorne Inc., Beverly, USA) was used to assess sperm motility. For the measurements, equal parts of extended semen and Tyrode's solution were mixed and analyzed after 10 minutes (0 hours) and 3 hours of incubation at 37 °C. To semen extended with egg yolk–free extenders, equal parts of Tyrode were added and measured after additional 10 minutes of incubation at 37 °C. In semen extended with Triladyl, Tyrode's solution containing 80 μg/mL Hoechst 33342 was used to stain sperm DNA to discriminate accurately between sperm and nonsperm particles (especially egg yolk components) [14] using the Ident Fluorescence Option "Full Analysis" of the IVOS II system. For each sample, a 20-μm-deep semen analysis Leja 2-chamber slide (Leja, Nieuw-Vennep, the Netherlands) placed on a prewarmed stage (37 °C) was filled with semen and a minimum of 1000 cells were analyzed in no less than eight randomly selected fields, with 30 frames acquired per field at a frame rate of 60 Hz. For further analysis, the percentage of rapid cells with average path velocity ≥50 μm/s was considered as sperm motility.

2.2.2. Chemicals and reagents

Chemicals used for the preparation of Tyrode's solution, TNE buffer (0.01 M Tris, 0.15 M NaCl, 1 mM EDTA, pH 7.4), and acridine orange (AO) staining buffer (0.2 M Na2HPO4, 1 mM EDTA, 0.15 M NaCl, 0.1 M citric acid, pH 6.0), the fluorescein isothiocyanate–conjugated lectin from *Arachis hypogaea* (FITC-PNA), propidium iodide (PI), Hoechst 33342, and Triton X were purchased from Sigma Aldrich Co. (Buchs, Switzerland). 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was obtained from Life Technologies Europe B.V. (Zug, Switzerland), whereas AO was purchased from Polysciences Europe GmbH (Eppelheim, Germany). Fluorescent probes were diluted and used for sperm staining in form of working solutions with following concentrations: 2.99 mM PI, 100 μg/mL FITC-PNA, and 0.153 mM JC-1.

2.2.3. Plasma membrane and acrosome integrity and mitochondrial membrane potential of sperm

Flow cytometric assays regarding plasma membrane and acrosome integrity (PMAI) as well as mitochondrial membrane potential were performed using a Cell Lab