Animal Reproduction Science xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Animal Reproduction Science

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

Impact of holding and equilibration time on post-thaw quality of shipped boar semen

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ARTICLE INFO

Keywords:
Boar semen
Cryopreservation
Equilibration
Holding time
Shipping

ABSTRACT

Cryopreservation of boar semen is of growing interest for breeding companies. Overnight-shipping of pre-diluted ejaculates to specialized laboratories offers a practicable method, but requires fine-tuned protocols. In this study, the impact of holding post shipping at 17 °C for 2 or 24 h (n = 10 samples) and of equilibration in lactose-egg yolk extender without glycerol at 5 °C for 2, 4, 24 or 48 h (n = 11 samples) before freezing was investigated. Sperm-rich fractions of ejaculates from 21 mature Pietrain boars were collected at a single boar stud. After pre-dilution (1 + 1, v:v) with Beltsville thawing solution, samples were sent to the laboratory. Temperature profiles during transport and initial equilibration time were recorded. Semen quality post-thaw (PT) was evaluated using CASA and flow cytometry. Holding of 2 h after shipping resulted in higher sperm motility (P = 0.013) and beat cross frequency (BCF; P = 0.047) compared to 24 h. Differences between both groups vanished with prolonged incubation at 38 °C PT. Equilibration at 5 °C for 4 h yielded the highest motility and BCF, whereas the equilibration for 48 h impaired sperm motility. Membrane integrity, mitochondrial activity and DNA fragmentation index were not affected by any protocol modification. In conclusion, processing of pre-diluted boar semen shipped overnight within 2 h after arrival at the laboratory is preferred to 24 h of additional holding at 17 °C. Extending the equilibration period in lactose-egg yolk extender without glycerol at 5 °C from 2 h to 4 h before freezing is recommended.

1. Introduction

Demand on freezing valuable boars' ejaculates for international trade and gene banking is high (Men et al., 2012; Knox, 2016). Worldwide less than 1% artificial inseminations (AI) in pigs are performed with frozen-thawed semen (Rodríguez-Gil and Estrada, 2013). Due to the composition of sperm membranes with high protein to phospholipid and low cholesterol to phospholipid ratios, boar spermatozoa are highly sensitive to low temperatures. Chilling injury is expressed as loss of motility, membrane integrity, mitochondrial activity and other sperm functions (Parks and Lynch, 1992; Maxwell and Johnson, 1997). Cryopreservation protocols for boar semen are delicate and complex in order to minimize sperm damage during processing and consequently are often not practicable in the daily routine of AI stations. Shipping of pre-diluted ejaculates to a central personally and technically well-equipped laboratory would allow freezing under ideal conditions thus offering a feasible, cost-efficient method for breeding companies. Overnight shipping inevitably introduces a holding time of extended semen for approximately 24 h at 17 °C before further processing.

The effects of holding time at 15–17 °C prior to cryopreservation have been discussed with different outcomes. It is assumed, that during holding time an alteration of the plasma membrane lipid composition and protein phosphorylation takes place, rendering the

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http://dx.doi.org/10.1016/j.anireprosci.2017.10.014
Received 14 August 2017; Received in revised form 20 October 2017; Accepted 24 October 2017

spermatozoa less susceptible for cold shock (Tamuli and Watson, 1994; Casas and Althouse, 2012). While Yeste et al. (2014) reported an improvement of lots of sperm quality parameters, other studies revealed negative effects on sperm kinematics and fertility when holding time was prolonged from 3 to 24 h (Tomas et al., 2014). A number of other researchers could not detect any differences between varying holding times before freezing (Kong et al., 2012; Gale et al., 2014). Noteworthy, during transport mechanical effects impinge on semen samples. Recently it was shown, that even gentle agitation of liquid preserved semen samples may be harmful to sperm quality (Schulze et al., 2015). Thus, shipping-associated stress factors might influence the response of boar sperm to cooling stress which so far has not been studied.Common freezing protocols contain a further holding step at 5 °C to equilibrate sperm in cooling or freezing extenders at a lower temperature. In bulls, extended equilibration in yolk-extenders at 4–5 °C of about more than 24 h influences sperm quality positively (Griga, 2008; Fleisch et al., 2017) and therefore has become popular in many AI centers. During this period, lipid uptake and metabolism can stabilize plasma membrane structure of spermatozoa (Maldjian et al., 2005; Bergeron and Manjunath, 2006; Svetlichnyy et al., 2014). For boar semen, in general an equilibration period of 2 h is used (Yi et al., 2002). We assume, that this period can be extended when equilibration is done in glycerol-free cooling extender, but not in freezing extender which commonly contains a final concentration of 2–3% glycerol. Thus, spermatozoa will not be exposed to the toxic effects of glycerol for a long period (Fuller, 2004; Macias Garcia et al., 2012; Sieme et al., 2016). At present, the effects of long-term equilibration times in shipped boar semen are not known.

The present study was designed to adapt a currently used cryopreservation protocol to pre-freeze conditions associated with pre-dilution and overnight transport at 17 °C. The aims were to evaluate the impact of holding-time of shipped boar semen at 17 °C after arrival in the central laboratory (Experiment 1) and to depict the effect of short- and long-term equilibration in cooling extender at 5 °C (Experiment 2). Overall, it is hypothesized that an adapted protocol offers breeding companies an improved possibility to freeze boar semen in external specialized laboratories.

2. Material and methods

2.1. Chemicals and extenders

All chemicals used were of analytical grade. They were purchased from Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) and Sigma-Aldrich (Taufkirchen, Germany). Transport extender (Beltsville thawing solution, BTS) and thawing extender (Androstar® Premium) were purchased from Minitüb (Tiefenbach, Germany). Cooling extender was composed of 20% egg yolk and 80% lactose-solution (310 mM). Freezing extender consisted of 92.5% cooling extender, 1.5 mL Orvus ES Paste and 6.0 mL glycerol.

2.2. Experimental design

Two independent split sample experiments using different ejaculates and random sampling were performed. For each experiment, three single shipments within six weeks in winter took place. The first experiment (Exp. 1, n = 10) was conducted to study the effect of an additional holding time at 17 °C of 1 + 1 (v:v) pre-diluted ejaculates after 20 h of overnight transport to the processing laboratory located at the Institute for the Reproduction of Farm Animals Schönow e.V. (IFN). After arrival, holding time (HT) of semen samples in transport extender was either 2 or 24 h (HT2, HT24). Holding during transport and after arrival in the laboratory summed up to a total holding time of 22 and 44 h, respectively (THT22 and THT44). Direct proceeding after arrival was not done as ejaculates first had to pass quality control, which took about 2 h. The second experiment (Exp. 2, n = 11) was performed to evaluate the influence of short (2 and 4 h) and long (24 and 48 h) equilibration time (ET) in standard lactose-egg yolk cooling extender at 5 °C on boar semen quality post-thaw (ET2, ET4, ET24 and ET48).

2.3. Animals, semen collection and transport

Single ejaculates of 21 mature Pietrain boars of proven fertility, aged 1–5 years, from a single boar stud in southern Germany were included in the study. All boars were routinely used for semen production and housed individually in straw-bedded pens according to the European Commission Directives of Pig Welfare. Sperm-rich fraction of the ejaculates was collected once a week using the gloved-hand method. Only ejaculates meeting the following requirements for commercial AI were used: semen concentration $\geq 200 \times 10^6$ spermatozoa/mL, raw semen motility ≥70% and ≤25% morphological abnormal spermatozoa. All samples were diluted isothermally 1 + 1 (v:v) with transport extender, filled into QuickTip FlexiTubes® (Minitüb, Tiefenbach, Germany) and manually sealed low-air. Then, they were packed in isolated boxes and sent to the IFN. Transport duration was about 20 h.

2.4. Semen processing

Upon arrival, subsamples for semen evaluation were collected from every pre-diluted ejaculate. All samples were split into two (Exp. 1) or four (Exp. 2) groups and put into a climate-controlled device (temperature 17 °C) for the duration of holding time. After holding for 2 or 24 h in Exp. 1 or 2 h in Exp. 2, samples were centrifuged (Rotanta 460R, Hettich, Tuttlingen, Germany) at 17 °C for 3 min at 2400 × *g*. The supernatant was discarded and semen concentration was adjusted to 1.5×10^9 spermatozoa/mL with cooling extender. Then, samples were placed in a climate-controlled cabinet at 5 °C for 2 h (Exp. 1) or 2, 4, 24 or 48 h (Exp. 2) for equilibration. Subsequently, final dilution with freezing extender to 1.0×10^9 spermatozoa/mL was done. Samples were filled into 0.5 mL medium straws (Minitüb, Germany) using an automated filling device (MPP Uno, Minitüb, Germany). Immediately after filling,