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The effect of extender, method of thawing, and duration of storage on *in vitro* fertility measures of frozen–thawed boar sperm

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ABSTRACT

Frozen–thawed boar sperm (FTS) has reduced *in vitro* and *in vivo* life span compared to liquid semen. Experiments tested whether extenders, thawing procedures, and storage temperatures could extend the fertile life span of FTS. Experiment 1 tested the effect of six extenders on postthaw motility (MOT) and viability (VIA). Straws from boars (n = 6) were thawed, diluted into each extender, and evaluated at 20, 60, and 120 minutes. There was a trend (P = 0.08) for an extender-by-time interaction for MOT and effect of extender and time for MOT (P < 0.0001) and extender (P = 0.10) and time (P < 0.0001) for VIA. Experiment 2 evaluated the effect of temperature and time of thawing on *in vitro* fertility at intervals after thawing. Straws (0.5 mL) from different boar ejaculates (n = 15) were thawed at 50 °C for 10, 20, or 30 seconds or at 70 °C for 5, 10, or 20 seconds and evaluated at 5, 30, and 60 minutes. There was an effect of thawing treatment on MOT, VIA, and ACR (viable sperm with intact acrosomes, P < 0.0001) and an effect of time of evaluation (P < 0.0001) on MOT and ACR. Thawing at 70 °C for 20 seconds reduced (P < 0.05) MOT, VIA, and ACR compared to other treatments. Experiment 3 tested the effects of storage temperature and time after thawing using 20 ejaculates. Samples were thawed, diluted, and allotted to storage at 17 °C, 26 °C, or 37 °C with evaluation at 2, 6, 12, and 24 hours. There was a storage temperature and time effect and an interaction for MOT and VIA (P < 0.0001). Storage at 17 °C and 26 °C increased (P < 0.05) MOT over all times (38.5%) compared to 37 °C (26%), whereas MOT was reduced at intervals. Viability was also greatest with 17 °C and 26 °C compared to 37 °C and was also affected by time and decreased with time. These results indicate that FTS can be held at 17 °C or 26 °C for up to 2 hours before use and would allow for preparation of multiple doses. These data suggest *in vitro* fertility of FTS is affected by extenders, thawing, and storage.

1. Introduction

In modern swine production, there is limited use of frozen–thawed boar sperm for artificial insemination because of the variable pregnancy rates and litter sizes when used for breeding [1,2]. The shortened life span of frozen–thawed boar sperm in comparison to liquid extended semen has been documented *in vivo* and *in vitro* and is assumed to be the cause of lower fertility when used in artificial insemination [3–5]. It has been hypothesized that postthaw handling procedures may have large implications for postthaw boar sperm quality measures such as motility, viability and acrosome status and could therefore impact *in vivo* fertility.

The *in vitro* motility and viability of boar sperm have been reported to be influenced by various extenders and

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components for liquid [6,7] and frozen sperm [2,8–10]. Because of considerable variation in freezing and thawing methodologies, there is no clear evidence on whether specific postthaw extenders could improve cryopreserved sperm fertility. Cryopreserved boar sperm is packaged in plastic straws or plastic bags of 0.25 to 5 mL volumes [1,11–14]. In some research studies, improved postthaw quality was observed in lower-volume straws and plastic bags (0.25–0.7 mL) because of the smaller surface area-to-volume ratio, which allows for more uniform freezing and thawing [11,15,16]. Because of the discrepancies in freezing volume, surface area, and surface area-to-volume ratio, different thawing temperatures and durations have been explored for sperm packaged in different forms. Although thawing was initially performed at 37 °C [17], higher thawing temperatures of 50 °C [16,18] and 70 °C [19] were chosen to minimize the exposure time of sperm cells to potential ice crystal and osmotic damage. As a result, thawing duration differed with lower-volume straws (0.25–0.5 mL) which were thawed at 10 to 20 seconds at 50 °C [16,20] or 5 to 10 seconds at 70 °C [19], whereas greater-volume straws (5–7 mL) were thawed for 40 to 50 seconds at 50 °C [18].

Although there has been extensive research on the importance of prefreeze semen handling [2,21,22], cooling rate [23,24], freezing rate [19,25], and thawing rate [26,27], there is limited information on many aspects of postthaw semen processing. This includes information on diluents, temperature and time of thawing, temperature and duration of storage on the fertile life span of frozen–thawed boar sperm.

To determine the effects of postthaw semen handling, three experiments were performed to evaluate the effect of different extenders, method of thawing, and storage on measures of *in vitro* fertility for frozen–thawed boar sperm.

2. Materials and methods

2.1. Boar sperm cryopreservation

2.1.1. Semen collection, freezing, and evaluation

The protocol for use of animals in these experiments was approved by the University of Illinois's Institutional Animal Care and Use Committee. Semen was obtained from purebred boars that were in commercial boar studs and routinely collected for production of liquid semen. Ejaculates were frozen using a modified procedure [23] that has been previously described [28]. Briefly, ejaculates were collected and diluted at a ratio of 1:1 with 37 °C extender (Modena; Swine Genetics International, Cambridge, IA, USA) and slowly cooled to 17 °C before overnight shipping to the University of Illinois, Urbana, IL, USA. On arrival, samples were evaluated for motility and concentration and processed if motility was 80% or greater. Samples were centrifuged for 12 minutes at 800 × *g* at 17 °C, and the supernatant was aspirated. The sperm pellet was resuspended with the Androhep CryoGuard Cooling Extender (MOFA, Verona, WI, USA) to a concentration of 2.8×10^9 sperm/mL. Sperm were held at 5 °C for 2.5 hours and diluted to a final concentration of 1.4×10^9 sperm/mL with Androhep CryoGuard Freezing Extender (MOFA). Straws (0.5 mL) were filled using a semiautomatic filling and sealing machine. Straws were placed into the IceCube controlled rate freezer (MOFA) at 2 °C and processed using the following freezing curve: step 1: 2 °C to −4 °C in 3 minutes (2 °C/min), step 2: −4 °C to −30 °C in 0.87 minutes (29.9 °C/min), step 3: −30 °C to −25 °C in 1 minute (5 °C/min), and step 4: −25 °C to −140 °C in 11.5 minutes (10 °C/min). After the completion of the freezing program, the straws were placed into containers for storage in liquid nitrogen at −196 °C.

2.2. Experiment 1: Effect of thawing extender on measures of sperm fertility at intervals after thawing

The aim of this study was to determine whether different extenders influenced the motility and viability of cryopreserved boar sperm after thawing. Frozen–thawed samples were selected from cryopreserved ejaculates that had a 20-minute postthaw motility greater than 40% in the extender designed for cryopreserved boar sperm (Androhep CryoGuard [ACG] extender). The other extenders were chosen on the basis of their availability as short-term and long-term extenders. The short-term extenders chosen were Beltsville Thaw Solution (MOFA) and Modena (Swine Genetics International), whereas the long-term extenders were Preserv Xtra (ReproQuest, Fitchburg, WI USA), Duragen (Magapor, Zaragoza, Spain), and Vitasem LD (Magapor). The extenders were diluted to the specifications of the manufacturer and stored at 5 °C. The experiment was performed using semen from six different boars, and sperm were frozen in 0.5-mL straws that had been stored in liquid nitrogen for 12 to 36 months. One boar was tested in each replicate. Within a replicate, straws from a single ejaculate were thawed at 50 °C for 20 seconds and the contents were expelled into a single glass tube in a dry heating block at 37 °C. Aliquots of the sample were diluted at a ratio of 1:40 into one of the six different extenders in 1.5-mL polypropylene tubes. After 10 minutes, a vital stain (Viadent; Hamilton Thorne, Beverly, MA, USA) containing the fluorescent dye, Hoechst 33258, that had been diluted in each of the six different extenders, was added to a subsample for a final dilution of 20 μg/mL of stain and then incubated for 8 minutes at 37 °C. The vital stain was added to the different extenders at 1-minute intervals to avoid different incubation times. To avoid bias, the sequenced order for extender evaluation was rotated equally among the different extenders over replicates. After the incubation time, samples were analyzed using CASA (IVOS; Hamilton Thorne) using 20-μm Leja slides (Leja Products B.V., the Netherlands) for motility in five fields followed by assessment for viability in the same five fields using excitation of the fluorescent stain with a blue light-emitting diode and using the Viadent filter. Samples were evaluated at 20 (T0), 60, and 120 minutes after thawing.

2.3. Experiment 2: Effect of thawing temperature and duration of thawing on sperm measures at intervals after thawing

2.3.1. Semen and experimental design

In experiment 2, cryopreserved sperm from the ejaculates (n = 15) of 13 boars were used. The sperm had been stored at −196 °C for approximately 6 to 12 months before