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Original Research Article

Effects of different cryoprotectants and freezing methods on post-thaw boar semen quality

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ABSTRACT

The current study aimed to investigate the effects of different concentrations of glycerol (0%, 1%, 2%, 3%, and 5%) and dimethylacetamide (DMA: 0%, 1%, 3%, and 5%) on post-sperm quality characteristics following semen freezing in dry ice (D) or liquid nitrogen (N). Semen was collected from Duroc boars and was allocated to 32 treatment groups for cryopreservation. Analysis of post-thaw semen quality and fertility after artificial insemination (AI) was used to examine the combinatorial effects of different treatments. The best scores for post-thaw sperm motility, sperm viability, and sperm acrosomal integrity were observed in semen frozen in: (a) dry ice in the presence of 5% glycerol and no DMA (16D-treatment); (b) dry ice in the presence of 3% glycerol and no DMA (9D-treatment); and (c) liquid nitrogen in the presence of 3% glycerol and 1% DMA (10N-treatment), with no significant difference observed among these three treatments. The farrowing rates after AI with post-thawed semen after 9D- and 10N-treatments were 33% and 50%, respectively. To summarize, the results of the present study indicated that the freezing extender containing 3% glycerol in combination with the straw-freezing method using dry ice produced the best post-thaw quality parameters of boar semen. Combinations of glycerol and DMA did not enhance the cryosurvival of boar spermatozoa.

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1. Introduction

The advantages of using cryopreserved boar semen include long-term preservation of favorable genetic resources, long-distance transportation of valuable genetic material and guaranty to prevent the spread of pathogens [1]. Boar spermatozoa are highly sensitive to the freeze–thaw process, which causes considerable cell damage and leads to substantial reductions in farrowing rates and litter size. These factors are responsible for the limited use of frozen boar semen at the commercial level [1,2].

Routinely, semen is diluted in an extender containing cryoprotectants in order to minimize intracellular crystallization. Glycerol is the most commonly used penetrating cryoprotectant for cryopreservation of mammalian semen and effectively preserves post-thaw sperm function (motility, viability and acrosome integrity) [3]. Despite its benefits, some studies have reported low fertility rates when boar spermatozoa were frozen with extenders containing a high concentration of glycerol (4–8%) [4–8]. Recently, several amides, such as dimethylacetamide (DMA) and dimethylformamide (DMF) have been used as an effective substitute for glycerol in the freezing protocols for boar, stallion, rooster and rabbit semen [7,9–11]. Since most amides are hydrophilic by nature and have a lower molecular weight compared to glycerol, they may induce a limited osmotic damage to spermatozoa [12]. In addition, diverse chemical structures and hydrophilic nature of cryoprotectants might trigger diverse reactions with the sperm cells that could affect post-thaw semen quality [13–16].

The freezing and thawing rates also influence sperm cryosurvival [17,18]. An optimal cooling rate must therefore be determined to minimize cryoinjuries, which include the disruption of the sperm plasma membrane and DNA structure, induced by extensive intracellular ice formation as well as changes in the intracellular pH and ionic composition [17,19,20]. Our previous study demonstrated that favorable straw freezing methods for glycerol-based extenders were different from those with DMA, and the interaction that occurred between the cooling velocity and cryoprotectants affected post-thaw boar semen quality [8]. Furthermore, the experimental design of the current study was principally derived from our previous research [8], which showed that the addition of Equex STM paste to extenders containing 5% DMA or 5% glycerol could improve the cryosurvival of boar spermatozoa, and highlighted the beneficial effects of the straw freezing method with different cryoprotectants. However, there were low fertility rates in preliminary artificial insemination (AI) trials using extenders containing 5% DMA or 5% glycerol. Therefore, the aim of the current study was to evaluate the effects of different concentrations of glycerol (0%, 1%, 2%, 3%, and 5%) and DMA (0%, 1%, 3%, and 5%) as well as the straw freezing method (liquid nitrogen or dry ice) on post-thaw boar semen quality.

2. Materials and methods

2.1. Semen collections

The gel-free fraction of boar ejaculate (approximately 200–350 mL) was collected into a pre-warmed plastic container (38 °C) covered with gauze, using the gloved-hand technique. The ejaculates were collected from two or three Duroc boars, giving a total of seven ejaculates. Sperm motility was evaluated subjectively with a phase contrast optical microscope (Olympus BX50, Tokyo, Japan) at ×100 magnification. Only ejaculates with ≥70% motility were processed. Sperm concentration was calculated, using a haemocytometer (Marienfeld-Superior, Lauda-Königshofen, Germany) [21].

2.2. Semen cryopreservation

Immediately after collection, the ejaculates were diluted (1:1; v/v) in Beltsville Thawing Solution extender (BTS; Minitüb, Abfüll-und Labortechnik GmbH & Co. KG, Tiefenbach, Germany) [22] and transferred to the laboratory in a container (16 °C) within 60 min. On arrival at the laboratory, each extended semen sample was immediately centrifuged at 800 × *g* for 10 min at 16 °C. The supernatant was discarded and the sperm pellets were re-suspended (1:1; v/v) in a lactose-egg yolk extender (11% lactose solution (w/v) and 20% egg yolk, 0.5% Equex STM paste). Subsequently, each semen sample was divided in 32 equal parts for the treatment, stored in a container for 60 min at 16 °C and then further cooled for 120 min at 5 °C. Following cooling at 5 °C, the freezing extender containing glycerol (Sigma, St. Louis, MO, USA) and/or N,N-dimethylacetamide (DMA, Sigma) was added to the samples. The freezing extenders consisted of different concentrations of glycerol (0, 1, 2, 3 or 5%) and/or DMA (0, 1, 3 or 5%) yielding 16 samples to be frozen in dry ice (D-treatments) and 16 samples to be frozen in liquid nitrogen (N-treatments). The specific concentrations of glycerol and DMA in each D- or N-treatment are depicted in first three columns of Tables 2 and 3. Semen sample was diluted (2:1; v/v) in the freezing extender and packaged in 0.5 mL straws (400–450 × 10^6 spermatozoa/mL). The first set of cooled 16 semen samples (D samples) was frozen by placing straws horizontally at the surface of dry ice for 10 min, whereas the second set of 16 samples (N samples) were frozen by placing the straws horizontally at 3 cm above the surface of liquid nitrogen for 20 min. At the end, all frozen straws were stored in liquid nitrogen until required. For sperm assessments, the frozen straws were thawed for 20 s at 37 °C, and the semen samples were re-suspended (1:20, v/v) in a pre-warmed (37 °C) BTS extender.

2.3. Semen evaluation

Ten microliters of diluted semen were placed on a microscopic slide, covered with a cover glass (18 mm × 18 mm) and at least 10 microscopic fields were examined at ×200 magnification under a phase contrast optical microscope (Olympus BX50) equipped with a pre-warmed (38 °C) stage. Sperm viability and morphology were studied using nigrosin and eosin [23]. Morphological abnormalities of spermatozoa were classified as major or minor [24]. Only major abnormalities were recorded when both major (e.g., teratoid head, kinked mid-piece, double tails) and minor abnormalities (e.g., detached head, simple bent or coiled tail, terminally coiled tail) simultaneously occurred in a spermatozoon.

Acrosome integrity of fixed spermatozoa was assessed using a fluorescein isothiocyanate labeled peanut agglutinin