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Cryoprotectant optimization for sperm of diploid Pacific oysters by use of commercial dairy sperm freezing facilities

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Abstract

Although sperm cryopreservation has been practiced in aquatic species for more than 50 years, viable markets for frozen sperm do not currently exist for commercial aquaculture. The present study suggests that the use of commercial cryopreservation facilities used for dairy bulls could be a cost-effective approach to initiate commercialization of frozen sperm in aquaculture, and the oyster industry could become one of the early adopters. To prove the technical feasibility of the use of a commercial freezing facility, this study adopted dairy freezing methods and emphasized cryoprotectant optimization for sperm from diploid Pacific oysters *Crassostrea gigas* with specific cooling methods employed for use with bull sperm. Specifically, the present study evaluated dimethyl sulfoxide (DMSO) at 5, 8, and 10%, ethylene glycol (E-glycol) at 2, 5, 8, and 10%, and methanol at 2, 4, 6, and 8%. Each cryoprotectant with its optimal concentration was chosen for subsequent selection of an optimal cryoprotectant. Previous results showed propylene glycol (P-glycol) at 5% yielded higher percent fertilization than did PG at 10 or 15%. Therefore, 5% of these cryoprotectants were compared and the highest percent fertilization was obtained with methanol (49±29%), followed by E-glycol (42±15%), DMSO (31±18%), and P-glycol (22±12%). Extensive evaluation for single and combined cryoprotectants and their concentrations were studied in our previous trials on a research scale (reported elsewhere), and 6% methanol and the combination of 4% methanol and 2% polyethylene glycol (PEG; FW 200) were shown to consistently yield the highest percent fertilization. Our last commercial-scale experiment compared 6% methanol with the combination of 4% methanol and 2% PEG (MET/PEG) with 20 oysters. There was no significant difference for percent fertilization between 6% methanol (39±29%) and 4% MET/2% PEG (42±26%). These findings demonstrate the technical feasibility of adopting dairy freezing protocols in commercial application for oyster sperm, and also provide a template for future commercialization of sperm cryopreservation for other aquatic species.

Keywords: Cryoprotectant; Sperm cryopreservation; Commercial; Oyster; *Crassostrea gigas*

1. Introduction

Sperm cryopreservation began in aquatic species more than 50 years ago (Blaxter, 1953) with study since then of approximately 200 fish species (Rana, 1995; Tiersch, 2000) and 30 invertebrates (Gwo, 2000). Although these research efforts have yielded protocols

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that are being applied with varying levels of success, no markets for frozen sperm currently exist for application in aquaculture (Lang et al., 2003). Currently, human medicine and livestock agriculture are the only worldwide industries that have incorporated cryopreservation of semen into artificial insemination practices (Crister, 1998; Centola, 2002; Curry, 2000). The successful application of cryopreservation in the dairy industry is due to the many advantages associated with frozen bull semen: 1) economic benefits such as increased availability of semen and reduced transportation and holding costs; 2) ability of farmers to select bulls that exhibit the most desirable traits, and 3) facilitation of selective breeding and subsequent manipulation of the genetic makeup of dairy cows for increased milk production. Similar benefits could be applied to sperm cryopreservation in aquatic species, although markets for frozen aquatic sperm still lie somewhere beyond the early stages of commercialization (Caffey and Tiersch, 2000b). Based on the insights provided by the existing dairy model, economic and marketing analysis for the potential usage of frozen sperm from aquatic species revealed important factors that inhibit scaling-up to commercial production: 1) economic constraints (e.g., capital costs for required equipment and operating costs for supplies and labors) (Caffey and Tiersch, 2000a); 2) doubts concerning the benefits (e.g., aquaculture industries are not completely aware of the benefits associated with frozen sperm) (Boever, 2006) because evidence proving cryopreservation to be a more cost-effective option than traditional spawning is not available for aquatic species, and 3) lack of early adopters (e.g., producers of species with substantial economic impact, equipped with the necessary technical capabilities, and employing artificial spawning methods) (Caffey and Tiersch, 2000b).

If cryopreservation of fish or shellfish sperm is to be integrated into hatchery operations, one approach to overcome the economic barriers and to prove the effectiveness for aquaculture is the use of specialized cryopreservation centers such as dairy facilities (Tiersch et al., 2004). Years of research and refinement have resulted in a dairy industry that efficiently processes, stores, and tracks semen, making improved germplasm available for breeders. Use of existing dairy freezing facilities provides access to standardized procedures and expensive equipment such as labeling systems, automated straw fillers, bulk-freezing chambers, and storage and inventory capabilities. Therefore, hatcheries could be exempted from the initial investment (mainly the equipment capital and labor costs), and confine their investment to shipping, processing (freezing paid upon service), and storage expenses. Meanwhile, hatcheries would have the flexibility to determine production scale based on available budgets. This approach also provides the additional benefits of well-established methods for quality control, inventory, secure storage, and transport. Conversely, dairy facilities could benefit from the extra income resulting from providing services for aquatic species.

The realization of commercial production of frozen bull semen has been attributed to the worldwide development and application of artificial insemination (AI) in the dairy industry (Herman, 1981). This is because AI training made reproductive technicians receptive to collecting, transporting, storing, and using sperm, which in turn facilitated the later adoption of cryopreservation. Based on this, it was predicted that the logical beginning for commercial application of sperm cryopreservation would be with those aquatic species currently produced by artificial spawning methods (Caffey and Tiersch, 2000a,b). The Pacific oyster, *Crassostrea gigas*, fits into this category because artificial spawning is routinely practiced in oyster hatcheries, and sperm is dry-stripped, stored at 4 °C in the refrigerator, or shipped chilled for fertilization elsewhere (e.g., Dong et al., 2005a). In addition, the Pacific oyster has a world-wide market and certified (e.g., for quality, or disease-free status) frozen sperm would facilitate international distribution, especially to those more selective markets where import of seedstock or broodstock oysters is discouraged (e.g., Europe, Chile) or where direct production of triploid oysters by use of cryopreserved sperm from tetraploid oysters is desired (Dong et al., 2005a). Thus, oyster producers could be considered as potential early adopters for commercialization of frozen sperm, and this would be especially useful for high-intensity breeding programs in oyster industries.

It is worth mentioning that dairy freezing methods such as those used at the LSU T.E Patrick Dairy Improvement Center in Baton Rouge, Louisiana (detailed below) employ a chamber that can freeze 660 0.5-mL standard French straws within 8 min, and can be operated by technicians with minimal training. Although such a freezing curve may or may not provide optimal cooling rates for a specific aquatic species, its cooling rate (on average of 16 °C/min) is within a workable range of cooling rates for most aquatic species (a magnitude of 10, Leung and Jamieson, 1991; Zhang, 2004). This provides the logic for applying dairy freezing methods to sperm of aquatic organisms. In particular with oyster sperm, a wide range of cooling rates have been reported: from 1 °C per min (Hughes, 1973) to direct plunging in liquid nitrogen (Hwang and Chen, 1973).

Thus, the adoption of dairy freezing methods and the use of specialized cryopreservation facilities could be a