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Strategies for commercialization of cryopreserved fish semen

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ABSTRACT - Initial success in sperm cryopreservation occurred at about the same time for aquatic species and livestock. However, in the 50 plus years since then cryopreserved sperm of livestock has grown into a billion-dollar global industry, while cryopreserved sperm of aquatic species remains a research activity with little commercial application despite work in more than 90 species and more than 200 published reports. Most research work has focused on large-bodied culture and sport fishes, such as salmon, trout, carp, and catfish, and mollusks such as commercially important oyster and abalone species. However, only a few studies have addressed sperm cryopreservation in small fishes such as zebrafish, or in endangered species. Overall, this work has yielded techniques that are being applied with varied levels of success around the world. Barriers to expanded application include a diverse and widely distributed literature base, technical problems, small sperm volumes, variable results, a general lack of access to the technology, and most importantly, a lack of standardization in practices and reporting. The benefits of cryopreservation include at least five levels of improvements for existing industries and for creation of new industries. First, cryopreservation can be used to improve existing hatchery operations by providing sperm on demand and simplifying the timing of induced spawning. Second, frozen sperm can enhance efficient use of facilities and create new opportunities in the hatchery by eliminating the need to maintain live males, potentially freeing resources for use with females and larvae. Third, valuable genetic lineages such as endangered species, research models or improved farmed strains can be protected by storage of frozen sperm. Fourth, cryopreservation opens the door for rapid genetic improvement. Frozen sperm can be used in breeding programs to create improved lines and shape the genetic resources available for aquaculture. Finally, cryopreserved sperm of aquatic species will at some point become an entirely new industry itself. A successful industry will require integrated practices for sample collection, refrigerated storage, freezing, thawing, rules for use and disposal, transfer agreements, and database development. Indeed the development of this new industry is constrained by factors including the technical requirements for scaling-up to commercial operations during the transition from research, and the absence of uniform quality control practices, industry standards, and appropriate biosecurity safeguards.

Key words: Aquatic species, Cryopreservation, Germplasm, Repositories

Introduction

Fish farmers are beginning to look more often to genetic improvement for gains in production; however, improving the genetics of fish species can take a long time. It is difficult to keep track of individual males or females and thus the process of developing breeding stocks and improved lines could take a decade or more. At the same time, fish managers are looking for ways to protect endangered species. Conservation programs require large populations to ensure biodiversity but for threatened and endangered species the numbers of fish are steadily decreasing. Cryopreservation can help in both of these situations. The availability of frozen sperm is a proven technique for developing, maintaining, and distributing genetic improvement in livestock, and provides great unexploited potential for fish breeding. In addition the availability of frozen sperm allows conservation programs to make a genetic bank of many males and increases the potential breeding population size to ensure that proper genetic combinations are produced in breeding of endangered species. In this way development of a single technology -- cryopreservation -- can assist two great needs: improved aquaculture and conservation of threatened and endangered fish species.

Initial research success in sperm cryopreservation came more than 50 years ago for aquatic species and livestock. Since then cryopreserved sperm of livestock has grown into a near-billion-dollar global industry. However, cryopreservation of aquatic species sperm remains essentially a research activity that is just beginning commercial application. Most aquatic research work has focused on large-bodied aquaculture

and commercial fishes, such as salmon, carps, and catfishes. Other groups such as mollusks, represented by commercially important oyster and abalone species, have received a fair amount of attention as well. Only a handful of studies have addressed sperm cryopreservation in endangered species, or in small fishes, which are becoming increasingly important in biomedical research and in the aquarium trade. Overall, this work has yielded techniques that are being applied with varying levels of success around the world. However, barriers to expanded application include a diverse and widely distributed technical literature base, procedural problems, small and variable sperm volumes, variable results, a general lack of access to the technology, and most importantly, the lack of standardization in practices and reporting.

What is Cryopreservation?

The scientific research of cryobiology and cryopreservation can be traced back to the 1950s after the discovery of the protective qualities of glycerol for freezing of fowl sperm by Polge and colleagues (Polge et al., 1949). The first studies of fish sperm cryopreservation were published soon after by Blaxter (1953), and since then hundreds of scientific papers have been published on research around the world. Cryopreservation is a process where biological materials such as cells and tissues are preserved by cooling to very low temperatures, typically, -196°C (for liquid nitrogen), yet remain viable after later warming to temperatures above 0°C. In essence, cryopreservation involves the transfer of excess water from the inside of the cell to the exterior where it can form ice. Successful procedures balance the formation of ice crystals within the cells against excessive dehydration which damages cellular structures. For sperm cryopreservation, this process typically includes a series of steps: 1) sperm collection and dilution, 2) refrigerated (non-frozen) storage and shipping of samples, 3) examination of sperm quality by microscope, 4) addition of cryoprotectants, 5) packaging of the samples, 6) freezing, 7) frozen storage procedures, 8) thawing, 9) use for fertilization, and 10) production of early life stages for assessment of cryopreservation success (e.g., Tiersch et al. 2007). Protocol establishment involves evaluation and optimization of multiple factors at each step (e.g., the type and concentration for each cryoprotectant), and recognition of the interactions among the steps (e.g., between cryoprotectant and cooling rate).

The use of cryopreserved samples for aquatic species typically involves induced spawning by injection of gonadotropic hormones and the collection of unfertilized eggs. This allows for a variety of crosses such as the use of one male to fertilize eggs from several females, or for the eggs of one female to be fertilized by sperm from several males. This can lead to a breeding matrix where a group of select males can be mated with a group of select females to develop populations with distinctive traits. By assaying the parents for genetic markers by use of the tools of molecular biology or by careful assessment of phenotypes, breeders can develop broodstocks with enhanced characteristics, such as high growth rate or disease resistance. Such a process can also be used to cross two different species to develop hybrids with improved traits, although precautions should be taken to prevent the accidental escape of the hybrid fish into the wild.

Current Status of Fish Sperm Cryopreservation

For sperm cryopreservation to become a reliable, cost-effective tool for genetic banking in aquatic species, the overall process needs to be improved, and the approach needs to be integrated into an efficient large-scale platform that links with genetic and biological databases, long-term storage capabilities, inventory management, quality control, sample distribution pathways, biosecurity assurance, utilization and disposal practices, and a sound cryobiological foundation. At present, cryopreservation of embryos and eggs of fishes has met with little success, but larvae and oocytes of oysters have been successfully cryopreserved (e.g., Paniagua et al., 2001; Tervit et al. 2005). For fish, the published work is essentially limited to study of sperm, for which numerous reports have been devoted to optimizing specific components of cryopreservation procedures. However, aside from those factors mentioned above, other factors such as sample density, freezing container, starting temperatures, final temperatures (before plunging into liquid nitrogen), and dilution and cryoprotectant

removal after thawing can also affect results. Therefore, procedures must be tailored for each species or population based upon a thorough understanding of cellular properties.

A current problem in cryopreservation research is the lack of standardization within the scientific literature for aquatic species in each step involved in the process. Comparisons among different studies are difficult to perform and could be invalid in most cases due to the procedural and reporting variations across studies. This is especially true for measurement and reporting of sperm concentration which are not reported for the majority of published studies (Dong et al. 2007a). Optimization of protocols without standardization offers little value for the improvement of existing methods and results, especially for the future development of commercial application. Controversy and inconsistency would be reduced if more congruent approaches were utilized and results among various studies could be directly compared. Suggestions for improvement include the creation and widespread acceptance of standard references to assist in harmonizing terminology, and the development and utilization of standardized educational programs. Standardization of research practices and reporting could be accomplished through establishment of guidelines for publication of results. Once in place the guidelines could be made available to journal editors and reviewers to assist in evaluation of research reports.

Future Prospects and Models for Application of Cryopreservation in Aquatic Species

Cryopreservation research and application each require consideration of an interconnected series of activities and this involves more than simple freezing of samples. A successful program involves integrated practices for sample collection, refrigerated storage, freezing, thawing, rules for use and disposal, transfer agreements, and database development. This concept is usually described within the activities of a germplasm repository at a single facility. The application of cryopreservation offers many benefits. With respect to commercialization, the benefits of cryopreservation include at least five levels of improvements that address existing industries and the creation of new industries:

1) Cryopreservation, at a minimum, can be used to improve existing hatchery operations by providing sperm on demand and greatly simplifying the timing of induced spawning. This prevents the problem, for example, of collecting ripe eggs, but not having sperm available to fertilize them.

2) Frozen sperm can greatly enhance efficient use of facilities and create new opportunities in the hatchery by eliminating the need to maintain live males. Thus, potentially all of the resources in a hatchery, which are typically limited, could be diverted to use for females and larvae.

3) Valuable genetic lineages that currently exist, such as endangered species, research models, or improved farmed strains can be protected by storage of frozen sperm. This could be very important for species such as shellfish in which valuable broodstocks must be stored in natural waters.

4) Cryopreservation opens the door for rapid genetic improvement. Frozen sperm can be used in breeding programs to create new improved lines and shape the genetic resources available for aquaculture operations. A dramatic example of this potential opportunity is provided by the dairy industry, which relies almost entirely upon cryopreserved sperm to produce improvements in milk yields.

5) Cryopreserved sperm of aquatic species will at some point, likely within the coming decade, become an entirely new industry itself. The global market for dairy bull sperm is around 1 billion dollars each year. Large, highly valuable global markets for cryopreserved sperm of aquatic species are now on the horizon.

Sperm cryopreservation in aquatic species is only beginning to find application on a commercial scale. The development of this new industry is constrained by a number of factors including the technical requirements for scaling-up to commercial operations during the transition from research. This problem has been addressed by research in our laboratory over the past 10 years that documents the feasibility of utilizing commercial dairy cryopreservation facilities to provide a jumpstart for cryopreservation in aquatic species such as catfish and oysters (e.g., Lang et al., 2003; Dong et al., 2007b). The dairy bull industry provides a business model for developing commercial application for cryopreserved sperm of fish (Caffey and Tiersch, 2000a). In addition, industries such as this can provide

equipment, protocols, facilities, and distribution networks that can be adopted for use with fish sperm (Caffey and Tiersch, 2000b).

Other challenges for commercial development include disease concerns for sample transfers, pricing structures, and product quality control issues. The presence of a cooperative framework across species can assist finding solutions for problems such as these. A model for developing multi-species repositories for genetic resources can come from the National Animal Germplasm Program (NAGP) of the United States Department of Agriculture (USDA) formed in 2000 (website: http://www.ars-grin.gov/animal/). The NAGP is patterned after the well-established USDA National Plant Germplasm System. The NAGP has committees for beef and dairy cattle, swine, goats and sheep, poultry, and aquatic species. The Aquatic Species Committee brings together members from universities, industry, and federal agencies. A structure such as this could assist development of repositories within and among other countries. Indeed, a useful model for aquatic species everywhere is the development of an integrated repository system that incorporates a single or a few well-equipped, experienced central facilities that carry out most of the cryopreservation work using samples or broodstock sent to the facility (Caffey and Tiersch 2000a). Other facilities can serve as satellite repositories to protect backup samples, and as user endpoints for the samples such as hatcheries.

The cryopreservation research in our laboratory at the LSU Agricultural Center is intended to assist the transition from cryopreservation research to application through work on protocol standardization, gamete quality, sample labeling, and database development to provide a repository to protect genetic resources, including endangered species, and to assist in developing existing and future industries for culture of aquatic species. Work such as this needs to be done worldwide. Overall, beyond the initial development of facilities, procedures, and training of personnel, the largest practical constraints to realization of a cryopreservation industry for fishes is at present the absence of uniform quality control practices, industry standards, and appropriate disease transfer safeguards. The control of the movement of pathogens into and out of a facility or area is referred to as biosecurity. This topic will assume great importance in the future for use and transfer of frozen samples.

Future Needs for Commercial Application of Cryopreservation in Aquatic Species

In order for cryopreservation to assume a functioning role in assisting aquaculture production and aquatic species conservation it has to proceed beyond development of the initial technical requirements. A series of activities are required to be in place for full-scale application. These activities have not yet been fully implemented anywhere for any aquatic species. There are problems and barriers at each step, but getting started is usually the hardest part. The entry-level requirements for equipment, facilities, and training are high and force potential users to focus on technology development and technical problems. However after this hurdle is passed, the focus can shift to coordination of activities and realization of the great opportunities provided by cryopreservation. This would include establishment of high-throughput capabilities, which has recently been recognized as a focus for work by the US National Institutes of Health for biomedical models including fishes (workshop summary available at: http://www.ncrr.nih.gov/publications/#reports). A final phase of maturation in application would see cooperation and connections forming among governmental agencies, non-governmental organizations, academic institutions, and private companies. The major activities in this potential pathway for application would include:

1) Development of technical capabilities and facilities at well-funded and secure locations. 2) Establishment of training programs for procedural efficiency, and recruiting of personnel. 3) Development of appropriate biosecurity safeguards to control movement of pathogens in and out of facilities. 4) Development of a functioning storage repository, with rules for use and disposal of samples, and with appropriate security for basic services (e.g., electricity, liquid nitrogen, refrigeration capabilities, and aeration for aquaria and holding tanks) 5) Implementation of archival labeling of samples and the creation of robust databases capable of handling biological information concerning samples (including geographical information system (GIS) data on collections), and maintaining correct inventory and identification of sample locations. 6) Further development of capabilities computing and information transfer including the ability to interact and exchange information with other databases. 7) Increasing of the

sample processing capabilities to enable high throughput of samples. This would include installation and use of automated or semi-automated equipment for labeling, filling, and sealing of straws, and the procurement of commercial-scale freezing and storage capabilities. 8) After central facilities have developed strong operational capabilities, a sustained effort should be made to develop cooperation with other organizations and facilities. These relationships can include sharing of samples, capabilities and expertise. Efforts should be made to link cryopreservation with existing or planned activities such as fish sampling programs or cooperation with specialized hatcheries during spawning seasons. 9) To assist interactions among organizations, basic arrangements should be discussed and put forth as formal transfer agreements that can be negotiated and put in place to describe things such as responsibilities, rights, and ownership of samples. 10) Because different facilities will have different approaches there should be establishment of quality control protocols and standardization of labeling, terminology, reporting of results, and databases. 11) Essentially, individual repositories can at this point be linked by establishment of a full repository system, and end users of cryopreserved sperm, such as hatcheries and farms can interact with this system. 12) Coordinated regional activities can take place in individual countries or be administered across borders to encompass river systems or ecosystems. 13) Establishment of pricing structure, marketing, and business practices will be necessary for commercial investment.

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