ANCILLARY SCIENTISTS SYMPOSIUM—PS5322

Avian Genetic Resource Banking: Can Fish Embryos Yield Any Clues for Bird Embryos?[1]

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ABSTRACT Cryopreservation of avian germplasm is becoming better understood and more commonly practiced. However, one area that would be of great benefit for genome resource banking is the preservation of avian embryos. Little is know about the cryobiology of avian embryos, and they have never been successfully cryopreserved. However, it is likely that they share many of the challenges of other yolk-filled multicompartmental embryos. For example, the fish embryo has 1) a large overall size, resulting in a low surface-to-volume ratio, which retards water and cryoprotectant efflux/influx; 2) large-sized cells, such as the yolk, which could increase the likelihood of membrane disruption by intracellular ice formation; 3) compartments, such as the blastoderm and yolk, with differing permeability properties; and 4) susceptibility to chilling injury. Both the avian and fish systems share many physical and anatomical properties, and it is predicted that some of the same permeability barriers would exist in both as well. Although the systems are similar, some of the goals, and thus the practices, to protect the genome may be quite different. One of these major goals in avian developmental biology is to produce chicken:chicken transgenic animals, especially those with germ line transmission. Producing efficient germ line transmissions and being able to cryopreserve these transmissions would be extremely beneficial to both basic and agricultural science. This could be accomplished through the cryopreservation of embryonic gonadal tissue followed by grafting into a host. The gonadal/tail-graft system would provide an advantage for cryopreservation because it is small (in comparison with the whole embryo), has fairly uniform tissue, and contains the essential primordial germ line cells capable of recreating the genetic line of interest. Moreover, because the chicken is such a robust model for most other avian species, the cryopreservation of the gonadal/tail-graft may potentially open up similar treatments for other commercially important species.

Key words: fish embryo, avian embryo, cryopreservation, genome resource banking, gonadal tissue

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INTRODUCTION

One of the first tissues ever cryopreserved was avian semen, which was cryopreserved using glycerol (Polge, 1951). Glycerol typically has been considered the best cryoprotectant for avian semen cryopreservation, but enigmatically, glycerol produces contraceptive effects in the female reproductive tract (Hammerstedt and Graham, 1992). However in the past few years, sperm cryopreservation for avian species has become better understood and more successful, producing excellent fertility (Chalah et al, 1999; Long and Kulkarni, 2004; Woelders et al., 2005). Given this increased fertility of females with cryopreserved sperm (Woelders et al., 2005), genome resource management for avian species is now becoming a reality.

One area that remains very difficult, yet highly desirable for genetic resource banking is the cryopreservation of avian embryos. Our laboratory at the Smithsonian National Zoological Park has been working on fish embryo cryobiology (zebrafish) for many years, and it is likely that some of the issues and rewards surrounding avian embryo cryopreservation parallel those of fish. Yolk-laden embryos, including those from fish, reptiles, birds, and amphibians, represent a class of complex, multicompartmental biological systems that have not been successfully cryopreserved. Over the past 50 yr, the fish embryo has presented a challenge to cryobiologists because of its interesting and complex physiology. Despite the lack of success in cryopreserving fish embryos, scientific efforts move forward because of the potential benefits of success to conservation of aquatic ecosystems and to humanity through increased food production. The availability of cryopreserved fish embryos, however, could also have a profound influence on medical research, aquaculture, and conservation biology.

Received October 10, 2005.

Accepted October 17, 2005.

[1]Paper for the Poultry Science Association Ancillary Scientists Symposium, July 31, 2005, Auburn, Alabama, "Conservation of Avian Genetic Resources: Current Opportunities and Challenges," organized and chaired by Dr. Muquarrab Qureshi.

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Developing techniques for the cryopreservation of teleost germplasm is timely, and the need is pressing. At present, aquaculture is largely dependent on wild fish populations or continuous maintenance of living cultures. Unfortunately, the availability and productivity of these systems is continually threatened by accidents, natural disasters, breeding failure, and disease. For example, valuable genetic lines created for productivity and disease resistance, such as transgenic fish and hybrids (Houdebine and Chourrout, 1991), must be maintained in live culture systems. These strains can take years to generate, which is costly in terms of space, maintenance, and research effort, and are subject to loss through genetic drift. Systematic germplasm cryopreservation can have a profound impact on aquaculture 1) by allowing the maintenance of large gene pools and reducing inbreeding, 2) by reducing pressure on wild populations from collection activities, 3) by maintaining a constant supply of animals (i.e., some animals are unavailable in the wild during certain times of the year), 4) by decreasing aquaculture costs by reducing the facilities needed, 5) by reducing the impact (e.g., contamination with antibiotics) of aquaculture sites on wild populations and food resources, and 6) by sustaining productivity by minimizing the impact of live culture failures resulting from human error, natural disasters, breeding failure, and epidemics.

In addition to the important scientific and direct applications to aquaculture, cryopreservation of teleost embryos would have considerable conservation benefit to endangered species. Of the estimated 24,600 fish species (Nelson, 1994), the greatest number and diversity of fish species inhabit the tropical regions (Wilson, 1988). Rapid destruction of tropical ecosystems is a global event, and within the next 25 to 50 yr, massive extinctions of endemic species within these areas are predicted (Janzen, 1988; Raven, 1988). Many freshwater communities in developing areas, including Lake Victoria, the rivers of Madagascar, and the Atlantic rivers of Brazil, are in critical condition from human encroachment, causing habitat degradation, and many endemic species face extinction. Although not as rich in diversity, aquatic ecosystems in developed areas have similar problems. In North America and Europe, salmon stocks are threatened by competition with hatchery stocks, habitat degradation from deforestation, air pollution-induced acidification, and exclusion from home-stream breeding habitats by locks and dams. The development of frozen or "insurance" populations would preserve genetic diversity and assist efforts to prevent the extinction of wild fish species in natural aquatic ecosystems (Ballou, 1992; Wildt, 1992; Wildt et al., 1993). Most importantly, these frozen populations would allow the time to reform natural resource policies so that endangered habitats are saved. Then, the embryos could be thawed, cultured, and released into the rehabilitated environments.

A systematic approach, using fundamental cryobiological principles, is the key to achieving such a practical goal. Most of the important technological innovations that have advanced the field of germplasm cryopreservation arose from a sound understanding of the mechanisms of cryodamage and cryoprotection (Mazur, 1970, 1984). Successful cryopreservation of germplasm must address several important issues, including 1) the intrinsic biophysical properties of the cells (e.g., membrane permeabilities and osmotic tolerance limits) and 2) determination of the procedural cryopreservation steps, based on the cell's biophysical properties, necessary to minimize cryodamage and maximize survival (Rall, 1993).

Several factors complicate teleost embryo cryopreservatio, including 1) a large overall size, resulting in a low surface-to-volume ratio that could retard water and cryoprotectant efflux/influx; 2) large-sized cells, such as the yolk, which could increase the likelihood of membrane disruption by intracellular ice formation (Mazur, 1984); 3) compartments, such as the blastoderm and yolk, with possibly differing permeability properties; 4) a semi-permeable membrane surrounding the embryo, such as the chorion, that may inhibit water and cryoprotectant influx/efflux (Wallace and Selman, 1990); and 5) potential susceptibility to chilling injury (Stoss, 1983, Zhang and Rawson, 1995). It is suspected that these may be similar for avian embryos, as well.

As stated earlier, fish embryos have never been cryopreserved. However, my colleagues and I think we know why. Although there is an outer fertilization envelop (similar to that of the developing bird) or chorion in the fish embryo, this is permeable to most solutions and only acts as a mechanical barrier. The chorion can be removed, and the fish embryo can be cultured successfully without it. Beyond that, we have found a major permeability barrier in the zebrafish embryo, the yolk syncytial layer (Hagedorn et al., 1996, 1997a, b, 1998). This layer physiologically blocks the entry of some cryoprotectants into the yolk and water's exit from the yolk, because of the yolk compartment's low water and cryoprotectant permeability. As a result of these permeability restrictions, conventional approaches to fish embryo cryopreservation result in the lethal destruction of the yolk syncytial layer (Hagedorn et al, 1998). Although this barrier can be circumvented by microinjecting cryoprotectant directly into the yolk (Janik et al., 2000), the need to remove water from the large yolk compartment remains. Because of the zebrafish embryo's large size (ca. 800 μm) and low water permeability, the dehydration time of the yolk and blastoderm is significantly longer than reported values for the mouse (Leibo, 1980), bovine (Ruffing et al., 1993), and permeabilized *Drosophila* embryos (Lin et al., 1989). Additionally, zebrafish membranes may have some unique osmotic properties. Therefore, to achieve successful cryopreservation of the fish embryo, the challenge is to enhance membrane permeability to allow a more rapid efflux/influx of water and cryoprotectants. To accomplish this, we modified the membranes of the fish embryo with water channels, or aquaporins, to enhance the water and cryoprotectant permeability of their membranes (Hagedorn et al, 2002).

Cryopreserving Avian Systems

Both the avian and fish systems share many physical and anatomical properties, and it is predicted that some of the same permeability barriers would exist in the bird, as well. Although the systems are similar, some of the goals, and thus the practices, to protect the genome may be quite different. The great strength of the avian system is the large quantity of basic developmental biology that is known about the chicken. One of the major goals in avian developmental biology is to produce chicken:chicken transgenic animals, especially those with germ line transmission (Mozdziak and Petitte, 2004; Mozdziak et al., 2005). From a practical point, many of the commercial poultry lines are inbred. Those that are not have the same problems that aquaculture has with the fragility of the stocks being maintained in live culture. Producing efficient germ line transmissions and being able to cryopreserve these transmissions would be extremely beneficial to both basic and agricultural science.

I believe the solution to all of these issues has already arisen from the elegant work of Petitte et al. Those researchers clearly indicate that pluripotent embryonic stem cells can differentiate into all somatic cell types and the germ line (Petitte et al., 2004). Although these cells can be found in the blood, they also segregate in early gonadal tissue clearly identifiable in the developing embryo (Modziak et al., 2005). As a model system, chickens also appear to be able to accept grafts fairly easily and can be cultured successfully afterward (Gimario et al., 2003).

From a cryobiologist's point of view, taking this aforementioned body of work together, one of the most interesting areas for potential germ line preservation for avian species is the embryonic gonad. Instead of trying to remove and harvest primordial germ cells from this tissue, I would suggest another process for potential cryopreservation. Once the primordial germ cells have migrated to the gonad, I would suggest removing the entire tail, including the gonad and remaining somites. A team of cryobiologists and avian biologists could then investigate the cryobiological properties of this tissue. If the cryopreservation were successful (i.e., the cells remain intact and alive after freezing), then the entire tail could be grafted back into a chicken strain of interest to showcase the germ line transmission.

Trying to cryopreserve the entire avian embryo (yolk and all) would be a daunting task. Aside from other issues, the surface-to-volume problem of the avian embryo would pose an enormous barrier to the movement of solutes and cryoprotectants to effectively protect the cells from freezing damage. Thus, the gonadal/tail-graft system would provide an advantage for cryopreservation because it is small (in comparison with the whole embryo), has fairly uniform tissue, and contains the essential primordial germ line cells capable of recreating the genetic line of interest. Moreover, because the chicken is such a robust model for most other avian species, this tail-graft system may potentially open up similar treatments for other commercially important species.

If in fact the idea of using the tail-graft system has merit as a tissue for avian cryopreservation, then the same cryobiological approach that was used for the fish should be used for birds. Some important first steps will be examining cryoprotectant toxicity, how water and cryoprotectants move across the membranes of the cells, and the chilling sensitivity of the tissue. However, it is one thing to propose what may seem to be an easy solution for avian preservation from a cryobiological perspective, but this solution may be impractical from a developmental perspective. If this tail-graft system seems feasible both developmentally and practically, then perhaps the cryopreservation of avian lines is within the foreseeable future and will provide a powerful addition to our nation's agriculturally important genome resources.

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