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EMBRYO IMPORTATION AND CRYOBANKING STRATEGIES FOR
LABORATORY ANIMALS AND WILDLIFE SPECIES

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ABSTRACT

The transportation of embryos obtained from animal models, endangered species and nondomestic farmed animals (e.g., deer) can reduce/eliminate the need for shipping postnatal animals and thus has gained the interest of the biomedical and conservation fields. Efficient movement of germ plasm worldwide requires established cryobanks. Embryo cryopreservation has become a routinely successful technology for many species and efforts to develop usable cryobanks for many target species are ongoing. Recommended regulations for the movement of embryos from nontraditional (i.e. other than domestic livestock) species are nonexistent. Efforts are underway to establish domestic and international handling guidelines and to recommend suitable quarantine conditions to facilitate embryo importation. Further basic research on specific zona pellucida-pathogen interactions is encouraged to support embryo movement efforts.

Key words: embryo cryopreservation, importation, regulation, laboratory animal, wildlife

INTRODUCTION

Consideration of general guidelines and circumstances needing special attention to facilitate the international movement of embryos from laboratory animals and nondomestic species is timely and appropriate. The groundwork for international efforts began in France when the Office of International Epizootics (OIE; 23), based on extensive embryo-pathogen interaction research (8, 30, 31, 32), established guidelines to address movement of livestock embryos. The efforts of a group of scientists, many of whom actively serve in the International Embryo Transfer Society (IETS) and its Import-Export Committee and Subcommittees, were critical to the establishment of and later acceptance internationally by government regulatory officials of standardized embryo washing and pathogen testing procedures (1, 23, 31). These have to be regularly updated based on research and risk assessment information (40).

Laboratory animals maintained in defined environments require practical but effective guidelines to facilitate controlled embryo movements. Fewer papers have been published on laboratory animals than on livestock species, but the data strongly support the effectiveness of embryo transfer for eliminating microbial contamination (4, 5, 26, 27). Due to cost constraints and physiological limitations (e.g., embryo availability) of wildlife species, it is improbable that the same degree of scientific investigation will occur before formulating regulatory policies.

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Developing and promoting standardized embryo handling practices for these nontraditional species will be discussed and a rationale for establishing effective guidelines, based on the more limited information for nondomestic species, will be outlined. Data gathered for domestic species and some wildlife animal movement controls already in place may support developing guidelines, and thus extensive embryo-pathogen interaction studies may not be necessary to meet regulatory requirements for the international movement of this valuable germ plasm (i.e., embryos).

LABORATORY ANIMALS

Biomedical Research Demands and the Need for Embryo Cryobanking

Laboratory animals in the Rodentia and Lagomorpha orders (e.g., mice, rats, cotton rats, guinea pigs, gerbils, hamsters, rabbits) play a vital role in biomedical research worldwide. At the National Institutes of Health (NIH), many invaluable animal models of disease and normal biological processes are maintained in the Animal Genetic Resource (NIHAGR) for each of these species. As a stock center for laboratory animals, the NIHAGR routinely provides breeding nuclei to the biomedical research community and an embryo bank was established as an economical means to indefinitely preserve these rodent and rabbit genotypes. Embryo cryopreservation offers benefits for genotype management such as safeguarding against loss from disease, reproductive failure, natural catastrophe or genetic contamination. In the future, the cryobank will help minimize changes due to genetic drift by providing a resource from which defined genotypes can be periodically rederived. This will allow for restandardization of foundation breeding colonies. Also, rather than shipping postnatal animals, cryopreserved embryos would offer a simpler, more humane and easier method to transport requested models worldwide. An equally important impact of embryo biotechnologies would be the prevention of vertical transmission of some diseases.

The NIHAGR continually receives requests for laboratory animal models. Last year more than 2600 animals were shipped, including more than 1500 to laboratories outside of NIH, of which about 250 were shipped internationally. More requests from laboratories located outside the NIH could be filled if cryopreserved embryos were available. Postnatal animal shipments are halted during the climatic extremes of winter and summer. Cryostored embryos, however, could be shipped year round but require that experienced personnel are present to receive and process the embryos. Embryo handling skills and training in cryobiology also are needed to ensure embryo survival during warming and transfer.

At the NIHAGR, research models of known genetic origin and health status exist as closed breeding groups in defined living conditions in accordance with the "Guide for the Care and Use of Laboratory Animals" (25). Assisted reproductive technologies (ART) provide powerful tools to manage these genetic resources at stock centers, such as the NIHAGR, and to facilitate the movement of animal models to other facilities. For example, embryo cryopreservation supports long-term storage and eventual rederivation of particular genotypes, while embryo transfer provides a desirable alternative to hysterectomy to rederive specific pathogen-free (SPF) animals. As these biotechnologies become incorporated into laboratory animal breeding programs, embryos must be handled aseptically (29) to avoid any contamination or possible disease transmission by those imported and exported for biomedical research purposes.

Considering the inherent problems associated with shipping postnatal laboratory animals, and the advances in cryobanking technology, recommendations for handling embryos designated for movement are timely. Guidelines (16) developed at the NIH have been under review by the IETS Import/Export Committee and focus on the need to establish recommendations for all interested institutions. The proposed guidelines are being considered for inclusion in the future edition of the Manual of the International Embryo Transfer Society (31).

Shipment of cryopreserved embryos between research institutions, although not yet routine, does occur (20, 36). This approach is expected to be the preferred method for transporting NIHAGR models in the future. Because the NIHAGR maintains small foundation colonies, postnatal animals may not be available immediately. Banks of cryopreserved embryos will expedite requests, expand distribution and facilitate the international exchange of these valuable animal models by better coordinating animal supply/demand and minimizing the impact of environmental temperature on shipments.

Colony Health Status and Embryo-Pathogen Interactions

A screen for pathogens (especially zoonotics) is a typical part of routine health surveillance of facility colonies. Documenting pathogens is necessary before the shipment of postnatal animals. The health status of donor colonies is also a major concern for embryo shipments. Testing for the presence of certain pathogens at each institution would facilitate embryo transportation. Suggested agents to be tested for are listed below by species:

Mice: Cytomegalovirus, mouse hepatitis virus (MHV), mouse pox (ectromelia) virus, pneumonia virus of mice (PVM), GDVII/Theiler's encephalomyelitis virus, lymphocytic choriomeningitis virus (LCMV), lactate dehydrogenase virus (LDHV), Sendai virus, minute virus of mice (MVM), rotavirus (EDIM), polyoma virus, mouse adenovirus (MAAdV), mouse thymic virus (MTV), Hantaan virus, Reovirus-Type 3 (Reo-3), Tyzzer's bacillus, cilia associated respiratory (CAR) bacillus, Mycoplasma pulmonis, Salmonella sp., Pseudomonas aeruginosa, Citrobacter freundii and Pasteurella pnenmotropica.

Rats: Kilham's rat virus (KRV), H-1 virus, coronaviruses (RCV/SDAV), Sendai virus, PVM, Reo-3, MAAdV, LCMV, Hantaan virus, Mycoplasma pulmonis, Salmonella sp., Pseudomonas aeruginosa, Mycoplasma pulmonis and Pasteurella pnenmotropica.

Cotton Rats: Those listed above for both mice and rats, plus Simian virus-5 (SV-5).

Hamsters: LCMV, Sendai virus, PVM, SV-5, parvovirus (KRV), encephalomyelitis (GDVII) virus, Hantaan virus, Reo-3, Salmonella sp. and Pseudomonas aeruginosa.

Guinea Pigs: PVM, LCMV, SV-5, Sendai virus, Reo-3, Salmonella sp. and Pseudomonas aeruginosa.

Gerbils: LCMV, PVM, Sendai virus, Tyzzer's bacillus, Salmonella sp. and Pseudomonas aeruginosa.

Rabbits: Rabbit haemorrhagic disease virus, Pasteurella, Bordetella, Encephalitozoan, Treponema, Salmonella sp., Pseudomonas aeruginosa and coccidia.

Embryo transfer technologies have proven effective in eliminating pathogens from rodent and rabbit embryos. In mice, embryo washing/transfer procedures have been shown to remove cytomegalovirus (22), MVM (21), LDHV (22), MHV (4, 26) and Sendai virus (4, 24). Also, Mycoplasma pulmonis did not survive during in vitro culture of mouse embryos (15). Mycoplasma pulmonis, Kilham's rat virus, Sialodacryoadentitis/Coronavirus and REO-3 (27) and pinworms (Syphacacia muris, 35) were eliminated via transfer of rat embryos into SPF recipients. Bordetella bronchiseptica was eliminated from rabbits following transfer of embryos into Bordetella-free recipients (33). No data are available on other species. These technologies can be invaluable when exporting clean embryos from a colony with a compromised health status to an existing, clean colony at the importing institution.

Environmental Conditions and Suggested Embryo Handling Guidelines

Colony management systems and health status must be considered before attempting to bank and rederive pathogen-free embryos. These related factors determine both the complexity of the procedure(s) to derive clean embryos, and the quarantine strategy needed to ensure clean, imported animals without compromising the health status of the existing facility colonies. An

outline of colony management systems is included in the guidelines to provide a baseline against which to assess the risk of acquiring (importing) or shipping (exporting) embryos. This characterization of colony environmental conditions is a fundamental requirement for effective embryo import/export activities worldwide. The proposed guidelines (16) will suggest standardized terms to define colony housing conditions and the extent to which pathogens are present. They will be based on the experiences of the NIHAGR to-date providing research animal models worldwide.

Colonies are typically housed under conditions that can be placed into three broad categories: defined flora, conventional and undefined conditions. Defined flora typically would be germfree or barrier room (sterile) facilities and the animals would have a pathogen-free or microbiologically-defined status. Barrier colonies may differ from germfree (pathogen- and nonpathogen-free) in that animals might be inoculated with a cocktail of a non-pathogenic microbial flora. Embryos collected from defined flora animals would be considered pathogen-free and would require the least restrictive conditions to ensure that clean embryos are available. The recommendations for handling such embryos reflect those stated in the IETS Manual for embryos of domestic species (31), but for embryos in this category the need for washing is minimal. The health status of the donor colony must be documented in the cryobanking records so the importing institution can determine the quarantine requirements.

More restrictive measures are recommended for embryo recovery from conventionally housed (not maintained in sterile conditions) animals. These closed colonies, with a known health status, would probably need to follow the 10-step washing procedure and other protocols outlined in the IETS Manual, but incorporating trypsin treatment only if an enveloped virus is known to exist in the colony (30). Quarantine requirements would need to be stricter than for defined flora embryos due to the increased risk of contamination. Finally, colonies in undefined flora conditions would include animals from the wild or with an unknown health status. These would require the most restrictive embryo handling conditions, including health testing the breeder male and the donor females, use of a biological safety hood, testing flush medium for pathogens, 10-step washing possibly with trypsin treatment and quarantine in the importing institution following transfer of embryos.

These embryo handling guidelines (16), together with detailed records represent, at this time, the best approach to minimizing the risk of importing embryos contaminated with unwanted pathogens. These may play a role in supporting the in-house need of some animal facilities, and may in the foreseeable future be a requirement for exchange of animal models between institutions, domestic and international. Complying with the guidelines can reduce the risk of acquiring pathogens but further investigation of embryo-pathogen interactions is needed. The skill and knowledge of those collecting and handling embryos is critical to the success of this type of animal model exchange as well. As advances in the technologies occur, the guidelines should be modified to reflect the most current information.

NONDOMESTIC WILDLIFE AND FARMED SPECIES

Management of Germ Plasm and Infectious Disease Concerns

The loss of genetic diversity in free-ranging and captive populations has increased the urgency for strategic plans to ensure long term species survival. Application of reproductive biotechnologies to wildlife populations may be beneficial for preserving this essential bio- and genetic diversity (28). The merits and strategies for establishing cryobanks for nondomestic species threatened or endangered by extinction have been detailed previously (37). Germ plasm repositories will 1) interactively support species populations, allowing for the infusion of genetic diversity; 2) enhance captive breeding efforts and reduce risks of disease transmission by utilizing AI, embryo transfer and IVF; 3) reduce animal numbers needed to ensure high levels of genetic diversity, thus decreasing captive breeding costs and space requirements; 4) transfuse wild germ plasm into captive programs without removing animals from indigenous habitats,

thereby providing insurance against epidemics, natural disasters and social/political upheaval; 5) eliminate the risks and costs of animal transport; and 6) provide increased access to rare specimens. Transporting healthy live animals can cause undesirable levels of shipment-associated stress leading to an increased susceptibility to disease or death (11, 19). In contrast, the cryopreserved embryo suspended in a metabolic resting state is well suited to international movement. Following embryo transfer the fetus benefits from peri- and postnatal antibodies from the recipient that offer protection from local diseases (1).

The development of strategies for semen and embryo banking as well as a greater understanding of disease transmission in wildlife populations are supported by various international conservation organizations (38, 42). The spread of disease is of major concern (41) when transporting wildlife, especially when the point of origin harbors pathogens such as rinderpest, foot-and-mouth disease and theileriosis (19). Incidents of disease transmissions by translocated wildlife were recently summarized (19, 41). Furthermore, the zoological community identified high priority diseases at an International Conference on Implications of Infectious Disease for Captive Propagation and Reintroduction of Threatened Species (6). The legal implications of managing captive breeding and reintroduction programs for endangered species also were addressed (7). In attempting to move animals, health and welfare issues as well as controls imposed by the Convention on International Trade in Endangered Species must be considered. Many potential hazards of moving live animals can be attenuated or eliminated by shipping gametes or embryos. It is generally believed that the potential for disease transmission via embryo transfer is much less than via semen (i.e., AI) or by the actual movement of potentially diseased animals (29, 30). Washing procedures have been shown to remove many pathogens common to domestic and wildlife species from zona-intact livestock embryos. Such procedures also may prove effective for wildlife embryos.

Gamete and Embryo Movement Guidelines

The field of conservation biology, unlike animal agriculture, lacks the economic incentives to establish guidelines for obtaining and transporting wildlife embryos. However, as interest grows in the use of semi-domestic farmed animals, such as deer and camelids (9, 18), for agricultural production combined with an increased regard for species preservation, the need for such recommendations is increasing.

Regulatory officials in the United States Department of Agriculture (USDA) Import Division currently consider gamete and embryo importations on a case-by-case basis (Karis, pers. comm.). The practices defined in the OIE Animal Health Code (23) for bovine, ovine/caprine and swine ova/embryos will apply to all potential shipments of wildlife embryos. Strict sanitary procedures and accurate record keeping by a qualified team supervised by an accredited veterinary official are required. Categorization of wildlife diseases, unlike those of livestock, has not been attempted due to an absence of basic research but, because of an existing Permanent Post Entry Quarantine (PPEQ) policy for international live animal shipments such categorizations may not be necessary. A few US zoological parks, selected on a regional basis, have been granted PPEQ status. Before arriving at these zoos, animals are quarantined pre- and post-shipment for disease screening and health status certification. These animals are maintained permanently on the PPEQ zoo premises but F1 generation offspring (≥ 6 months old) may be moved after appropriate disease testing. Routine preventative clinical and disease screenings are performed on the PPEQ animals. Quality assurance programs, including record keeping, are implemented by a veterinary medical officer who makes periodic visits to the PPEQ facility.

The current PPEQ program provides a foundation for the international movement of nondomestic animal embryos. This approach was successful in France where early imports of large numbers of bovine embryos under similar quarantine conditions proved effective in preventing disease transmission (34). Embryo and gamete handling procedures and record keeping practices must adhere strictly to the IETS Manual (31) and must be supervised by an official veterinarian. All nondomestic donor animals must be isolated and appropriate samples

(e.g., blood, excrement, tissue and nasal secretions) screened for pathogens at least 30 days before and after gamete/embryo collection. Embryos have to be stored in the exporting country's quarantine facility (30-60 days) until approved for export.

USDA officials are also willing to consider semen importations using the above mentioned precautionary measures. Bovine herpesvirus-1 (BHV1) might be eliminated from sperm preparations by serial washing (14). Furthermore, some believe that sperm transfected with certain viruses (i.e., bovine viral diarrhea, BVD) produce embryos which degenerate before blastulation (13), representing an in situ control against transferring contaminated embryos. These studies provide evidence suggesting that semen can be decontaminated. Unfortunately, the current United States regulations do not accommodate IVM-IVF derived embryos generated from culled field sources (17) or acquired opportunistically from rare or endangered captive animals that die unexpectedly. Regulatory concerns arise from the unknown health status of the donor, and the possibility of intrafollicular transfection (2, 3, 12). As a result, standardized practices such as those recommended for bovine IVM-IVF embryo production in the OIE Animal Health Code (23) are advised when cryobanking these potentially valuable samples, pending future modifications in the regulations.

The development of regulatory guidelines to safeguard against disease transmission by livestock embryos resulted from extensive investigations of embryo-pathogen interactions conducted in the late 1970s and 1980s (8, 30, 31, 32). While similar studies for many nondomestic species are highly unlikely due to limited animal resources and research funds, studies using surplus wildlife eggs and embryos (e.g., spare zonae pellucidiae) could provide preliminary data on the effectiveness of embryo washing and on zona adherence of selected pathogens. For example, investigators at Agriculture Canada are evaluating zona adherence and pathogen transmissibility of *Mycobacterium paratuberculosis* to ova and embryos in free-ranging deer and elk, and are collaborating with USDA on similar studies with foot-and-mouth disease (Singh, pers. comm.).

Future technological developments may reduce concern about possible transmission of infectious agents with embryos and spermatozoa but, meanwhile, tissue and fluid sampling and storage should become a routine practice of wildlife scientists involved in applying ART. In addition to blood sampling of both parents, flushing or follicular fluids and samples from serial washing steps should be stored for subsequent tests for pathogens. For specimens retrieved from culled animal sources, tissue samples should also be preserved and physical examination of the animal performed, as previously summarized for free-ranging wildlife (10). Non-transferable embryos and unfertilized oocytes should be preserved for future tests as well. Such projects are both realistic and practical, and most importantly would provide an invaluable source of information. If the approved importation of IVM-IVF derived embryos from wild sources is ever to occur, such associated biological materials must be available.

By using the PPEQ system it may be possible to conduct in vivo-in vivo studies in the future to definitively document the effectiveness of standardized embryo handling practices. For the present, however, it is essential that scientists and embryo team veterinarians using ART adopt routine practices maximizing data collection, emphasizing aseptic handling procedures and maintaining detailed records as suggested in the IETS Manual (29, 31) and the OIE Animal Health Code (23).

Future Perspective

Although progress is being made in species conservation biology using ART (17, 38, 39), continuing research in the area of gamete and embryo cryobiology is essential (28). Concurrently, it is vital that wildlife reproductive physiologists and veterinarians adopt stringent practices to avoid disease transmission via transported materials while adapting guidelines that comply with existing governmental regulations for domestic species. The international

movement of laboratory animal and wildlife embryos requires the implementation of standardized sanitary practices (31) and a better understanding of embryo-pathogen interactions.

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