

U.S. Department of Health and Human Services
National Institutes of Health
Division of Program Coordination, Planning, and Strategic Initiatives
Office of Research Infrastructure Programs

Cryopreservation and Other Preservation Approaches for Animal Models Workshop

Session V. Long-Term Preservation Methods for Nonhuman Primate Models in Biomedical Research

July 15, 2025
Virtual Meeting

Final Report

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Executive Summary

The Cryopreservation and Other Preservation Approaches for Animal Models Workshop was held in six sessions to address topics related to cryopreservation and other preservation methods, including, but not limited to, (1) the needs and scientific status of cryopreservation and other preservation of gametes (sperm, oocytes, and embryos), reproductive tissues, larvae, and whole animals (for some species) and their production of live offspring after reanimation; (2) emerging cryopreservation and other preservation methods and technologies, as well as how to optimize and implement them; (3) methods, technologies, and infrastructure to assess the impact of intrinsic and extrinsic factors on the quality, efficiency, and success of cryopreservation and other preservation protocols and revival, including scalability and reproducibility; (4) the sharing of technologies, including hands-on training for cryopreservation best practices and training of next-generation scientists; and (5) the preservation and management of samples, from collection to utilization.

Session V addressed long-term preservation methods for nonhuman primate (NHP) models in biomedical research. The presentations covered new preservation and reanimation methods for gametes and gonadal tissues from large animal models, species considerations for developing and cryopreserving genetic models in NHPs, protocols and preservation of valuable genetics in rhesus macaques, improved methods for freezing marmoset sperm, cryopreservation of gametes and embryos in NHPs, advances and challenges of ovarian tissue cryopreservation in an NHP model, and fertility preservation in a rhesus macaque model of cancer survivorship. During the discussion, the workshop panelists and participants identified needs related to facility infrastructure, animal and storage costs, technology advancements, protocol optimization, translation between NHP species and to humans, the rigor and reproducibility of NHP studies, and the incorporation of new approach methodologies.

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Carol Hanna, Ph.D., Oregon National Primate Research Center
Jenna Schmidt, Ph.D., Wisconsin National Primate Research Center

Presenters
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Pierre Comizzoli, D.V.M., Ph.D., M.Sc., Smithsonian's National Zoo and Conservation Biology Institute
Carol Hanna, Ph.D., Oregon National Primate Research Center
Brian Hermann, Ph.D., The University of Texas at San Antonio
Kyle Orwig, Ph.D., University of Pittsburgh
Jenna Schmidt, Ph.D., Wisconsin National Primate Research Center
Alison Ting, Ph.D., Gaia Life and University of California, San Diego

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Workshop Report

Opening Remarks

Bettina Buhring, Ph.D., Office of Research Infrastructure Programs (ORIP), Division of Program Coordination, Planning, and Strategic Initiatives (DPCPSI), Office of the Director (OD), National Institutes of Health (NIH)

Dr. Bettina Buhring welcomed the participants to the meeting. She explained that this is one of six sessions of the Cryopreservation and Other Preservation Approaches for Animal Models Workshop sponsored by ORIP. The workshop is providing a forum for experts to discuss needs related to animal model preservation and emerging technologies to enhance the maintenance and sharing of animal models. Dr. Buhring expressed appreciation to the speakers and organizing committee for this session.

Researchers use nonhuman primate (NHP) models to address specific scientific questions across a wide range of disorders to study disease progression and develop new therapies. NHP models, however, are very resource intensive. Long-term preservation of NHP germplasm via cryopreservation or other methods can serve as a potential solution to reduce costs. The session addressed the current state of the field, as well as gaps and barriers in developing long-term preservation methods for NHPs.

Keynote Presentation: Developing New Preservation and Reanimation Methods for Gametes and Gonadal Tissues from Large Animal Models

Pierre Comizzoli, D.V.M., Ph.D., M.Sc., Smithsonian's National Zoo and Conservation Biology Institute

In his keynote presentation, Dr. Pierre Comizzoli discussed his work on preservation and reanimation methods for gametes and gonadal tissues from large animal models. He began by emphasizing that a single model is only an approximation of the condition under study. By exploring a broad range of species, researchers can better understand and address complicated mechanisms and issues related to the fertility of common animal species or humans.

Dr. Comizzoli emphasized that individuals from wild populations are genetically diverse and present different phenotypes. He explained that his team does not use wild populations as laboratory models for diseases; rather, the animals provide information that can inform such future studies. The concept of "One Reproductive Health" suggests that the reproductive health of species is linked due to shared environments and overlapping ecosystems, resources, and needs. Similarly, researchers share common needs related to germplasm preservation (e.g., new fertility preservation options for animals, field-friendly methods of preservation, sample storage at minimal cost).

Large animal models offer the potential to capture the diversity and complexity of mammalian reproduction. Dr. Comizzoli explained that mechanistic differences in reproduction exist among species, and only a small number of species are well described among all mammals. This variation includes fertility issues, hormonal effects, and requirements for *in vitro* culture conditions. Species-specific considerations for cryobiology include industrial germplasm cryopreservation methods, as well as differences in cryoprotectant tolerance and resilience to cold and freezing temperatures. Thus, lessons learned in one species might not be applicable to another.

Specific considerations related to gamete and gonadal tissue preservation in large animal models, such as ungulates and carnivores, include sample collection (e.g., volume, size) and sample transport temperatures. Diversity of various factors—including gonadal morphology and histology, sperm morphology and activation, and oocyte structural characteristics and cryosensitivity—also plays a role. Dr. Comizzoli underscored the importance of these factors and shared examples of these differences among various mammalian species. He identified gaps in the field, which include the need for nontoxic

cryoprotectants that can cross the cytoplasmic membrane, reagents that protect cells from freezing stress, improved methods for warming and reanimation, and research into novel approaches (e.g., drying and storage at ambient temperatures).

Dr. Comizzoli presented an example of his work in mitigating cryodamage through manipulation of epigenetics in germinal vesicles of a domestic cat model. These findings potentially can be applied to other species. Rapid warming has been shown to be key for maintaining integrity and preventing damage related to refreezing. Dr. Comizzoli's team has explored several methods to facilitate rapid warming, including incubation with gold nanorods before ultra-rapid freezing followed by homogenous rewarming via laser. This approach brings the tissue back to physiological temperature quickly, thus preventing ice nucleation.

Examples of temperature protection in nature can provide insight into new preservation options because many organisms display natural protection from dry, hot, cold, or high-salinity environments. Some organisms can suspend life at nonfreezing temperatures by removing most of the water from their cells. Dr. Comizzoli also shared examples of "xeroprotectants," which include intracellular production of trehalose, late embryogenesis abundant proteins, heat shock proteins, intrinsically disordered proteins, and antioxidants.

Dr. Comizzoli outlined the successful use of trehalose, a drying agent, for preservation of cat sperm, testicular tissue, oocytes, and ovarian tissue. Transcriptomic studies indicate that the main cellular and molecular damages and stresses induced by dehydration are different from those of cryopreservation, and they are likely easier to mitigate. Preservation strategies at ambient temperatures would provide easy processing, transport, and use of biomaterials at a low storage cost.

In conclusion, large animal models provide comparative value in their applications to both wild species and human fertility preservation. Work at the intersection of multiple disciplines will be necessary to develop new preservation solutions. Additionally, more studies are needed to understand and mitigate cryodamage in gametes and gonadal tissues, especially regarding proper warming and reanimation. Stresses induced by dehydration differ from those of cryopreservation, and dehydration techniques may present new opportunities for long-term storage at nonfreezing temperatures.

Discussion

- Dr. Jenna Schmidt, Session Co-Chair, inquired about the reproducibility of drying techniques across different species, as well as the technical expertise necessary to perform such techniques. Dr. Comizzoli responded that his team works primarily with the domestic cat model, but he has experience in applying these protocols to closely related species. He also has performed preliminary studies in NHPs and sheep. In general, training for these protocols is straightforward.

- Dr. Carol Hanna, Session Co-Chair, noted that cat oocytes have a higher lipid content than those of NHPs. She wondered whether this difference needs to be accounted for in NHP vitrification studies. Dr. Comizzoli agreed that the level of lipids necessitates additional considerations in this area. His team has attempted to polarize the lipid droplets in cat oocytes, but no significant difference was observed. Additional work on this topic has been performed in bovine models.

- Dr. Peter Koulen inquired about xeroprotection of mitochondria. Dr. Comizzoli described the complexity of ensuring the integrity of various organelles and noted that metabolic changes are present during both cryopreservation and dehydration. His team has previously attempted to slow the metabolism prior to preservation, but no significant effects were observed from these efforts.

Species Considerations for Developing and Cryopreserving Genetic Models in Nonhuman Primates
Jenna Schmidt, Ph.D., Wisconsin National Primate Research Center (WNPRC)

Dr. Schmidt presented on developing and cryopreserving genetic models in NHPs, with a focus on species considerations. NHP models share similar features to human neurophysiology, endocrinology, anatomy, immunology, and behavior. NHPs also display high genetic sequence homology to human sequence and have a proven track record in elucidating mechanisms of human disease and in developing effective and safe therapies for human use in the clinic.

Genetic NHP models can be either identified from naturally occurring mutations (e.g., phenotype to genotype, genotype to phenotype) or created by introducing mutations via genome editing (e.g., CRISPR). Dr. Schmidt highlighted her prior work in both approaches. She discussed considerations for genome editing in NHPs, which include selection of species (e.g., nucleic acid sequence, protein sequence, behavior, anatomy), gene editor construct design, selection of efficient guide RNAs, and other factors.

Genetic NHP models have been developed for various diseases, including achromatopsia, endometriosis, Krabbe disease, Batten disease, epidermolysis bullosa simplex, Bardet–Biedl syndrome, Pelizaeus–Merzbacher disease, Usher syndrome, and Alzheimer's disease. Dr. Schmidt identified challenges in expanding genetic models, which include obtaining the desired mutation, time for expansion of the animal model, animal receptiveness to breeding or reproductive techniques, animal care and veterinary support, and financial support.

Researchers are interested in cryopreserving gametes, embryos, tissues (e.g., testicular, ovarian), and cells (e.g., fibroblasts, blood cells). Reproductive considerations for obtaining gametes and embryos include sexual maturity, female cycle, breeding, ovulation, gestation, and applied reproductive technologies. Dr. Schmidt outlined unique considerations for specific NHP species.

Rhesus macaques receive routine genetic screening, and data are made available via a genomic database (i.e., the Macaque Genotype and Phenotype Resource [mGAP]). Various reproduction methods (e.g., semen collection and freezing, ovarian stimulation, *in vitro* fertilization [IVF]/intracytoplasmic sperm injection [ICSI], embryo cryopreservation, embryo transfer) are well established. Challenges associated with laboratory reproduction in rhesus macaques include seasonal breeding, acclimation of males to semen collection, and efficiency of embryo transfer into dams.

In contrast to rhesus macaques, cynomolgus macaques are not seasonal breeders. Mauritian cynomolgus macaques have restricted major histocompatibility complex haplotypes, which makes them desirable for simian immunodeficiency virus studies and transplant studies. Established laboratory reproduction methods include semen collection and freezing, ovarian stimulation, IVF/ICSI, and embryo transfer. Challenges include difficulty in visualizing and detecting menses, difficulty in semen collection, lack of optimized embryo culture conditions for Mauritian cynomolgus macaques, and limited references for embryo cryopreservation.

Common marmosets offer such strengths as pharmacological regulation of the ovarian cycle, ongoing genome sequencing, and capabilities for genomic screening for human disease–relevant mutations. Established laboratory reproduction methods include semen collection and freezing, ovarian stimulation, and IVF/ICSI. Limited references for embryo cryopreservation are available. Challenges include social housing groups, high infant mortality rates, lack of an optimal protocol for ovarian stimulation, technical challenges with embryo transfer, and blood chimerism.

Dr. Schmidt highlighted the future potential of cryopreserved cells for genomic characterization, *in vitro* modeling, derivation of germ cells, and somatic cell nuclear transfer. She emphasized that researchers

must carefully consider how well the genetic model will recapitulate the disease in humans; anticipate how disease phenotypes will require additional health care, behavioral management, or support and modifications; and consider how the model will be expanded and preserved. Challenges in this space include limited references for some technologies and species, reproducibility of the methods, and technical expertise. Cryopreserved cells can serve as a future resource.

Discussion

- Dr. Hanna asked whether different NHP species respond differently to different types of technologies. Dr. Schmidt noted that she has not performed this type of study yet, but differences are likely to be present.

- Dr. Hanna wondered about the potential for developing cryopreservation methods in additional NHP species beyond those highlighted during the presentation. Dr. Schmidt noted that she has not received such requests. Dr. Hanna remarked that other NHP species, such as the baboon, offer additional potential.

- Dr. Buhring inquired about the current status of generating large structural genomic mutations, such as hundreds of excess triplet repeat expansions. Dr. Schmidt noted that she has not pursued efforts in this area, and it will likely be a challenge.

- Dr. Koulen asked about unexpected events resulting from CRISPR–Cas9 editing (e.g., mutations, genetic damage). Dr. Schmidt remarked that her team is highly interested in this topic and has performed relevant studies in embryos. She now is testing Cas9 variants to improve fidelity.

- Dr. Brian Hermann wondered about potential methods for intrauterine and intravaginal insemination in marmosets. Dr. Schmidt responded that these techniques offer potential, but screening of cervical anatomy will be important in this species.

- Dr. Samia Silva de Castro asked what ovarian stimulation protocol Dr. Schmidt's group uses. Dr. Schmidt responded that for marmosets, her team typically uses follicle-stimulating hormone (FSH) plus human chorionic gonadotropin (hCG). For rhesus and cynomolgus macaques, she typically uses FSH, gonadotropin-releasing hormone antagonist, luteinizing hormone, and hCG.

Protocols and Preservation of Valuable Genetics in Rhesus Macaques
Carol Hanna, Ph.D., Oregon National Primate Research Center (ONPRC)

Dr. Hanna discussed protocols for genetic preservation in rhesus macaques. During her presentation, she discussed NHP models that are available to researchers, an R24 grant aimed at developing a repository of valuable NHP genetics, and long-term storage protocols that are in development at ONPRC. She explained that the most common NHP species for research include the rhesus macaque, cynomolgus macaque, and common marmoset. Other models include the Japanese macaque, pigtailed macaque, and baboon. ORIP's National Primate Research Centers (NPRC) Consortium provides researchers access to these NHP models.

Dr. Hanna is the co–principal investigator on an R24 grant to develop a national NHP embryo resource of human genetic disease models. She explained that this resource is intended to address several current issues in the research community. First, researchers may have access to a model but lack the resources to preserve and store the model for future work. Second, researchers may currently lack the funds for animal studies and thus need to preserve the models for later use. Third, researchers may need a model but lack immediate access.

The project is led by a team of contributing scientists from Oregon Health & Science University; the University of Washington; the University of Wisconsin–Madison; Texas Biomedical Research Institute; the University of Mississippi; and the University of California, Davis. The specific aims of this project are to (1) establish a national NHP genetic disease model gamete, embryo, and fibroblast resource and a public, searchable tool for the long-term preservation of valuable NHP disease models and accessibility to those models; (2) support a nationwide collection of NHP gametes and the generation of genotyped model embryos by providing training in state-of-the-art assisted reproductive technologies; and (3) promote widespread use of the NHP genetic disease embryo resource through systematic outreach efforts aimed at reaching investigators nationwide.

Dr. Hanna reviewed the resource's procedures for the collection of oocyte and semen samples, IVF (e.g., conventional, ICSI), embryo culture, and embryo biopsy. She explained that the resource coordinates with mGAP. mGAP provides access to genotypic data collected on ONPRC's pedigreed rhesus macaque colony and identifies animals with naturally occurring genetic mutations. The database of DNA variants is curated and heavily annotated and can be viewed through the mGAP genome browser or downloaded for external use.

ONPRC follows a workflow for services and onsite training, which is guided by the requester's technical capabilities. Additionally, the resource is working to develop a protocol for oocyte vitrification in rhesus macaques. Dr. Hanna emphasized that more work is needed for advancement in this area. She concluded by highlighting success rates on oocyte vitrification and warming, post-thaw survival, fertilization, cleavage, and progression to the blastocyst stage.

Discussion

- In response to a question from Dr. Schmidt, Dr. Hanna clarified that the NIH grant covers training support for researchers. These efforts involve the dissemination of protocols and techniques.

- Dr. Schmidt asked about the current status of embryo vitrification in the field. Dr. Hanna responded that a robust protocol is in place. Currently, many of the embryos being used are gene edited, which affects the vitrification results. She noted that the thaw rates are strong. Embryo transfer also must be considered, but an effective protocol has been established.

- Dr. Caroline Mantovani da Luz inquired about hypotheses that explain why vitrified, warmed, and fertilized oocytes fail to develop into blastocysts. Dr. Hanna remarked that these results likely relate to disruption of tubulin filaments and actin.

- Dr. Matthew Jorgensen wondered about the greatest challenges for achieving cryopreservation in other NHP species (e.g., vervets). Dr. Hanna stated that access to animals remains a significant challenge. She noted that ONPRC maintains a small baboon cohort for contraceptive studies; this cohort has also been used for vitrification studies. Dr. Schmidt added that obtaining funding for such studies is an ongoing challenge.

- Dr. Kyle Orwig remarked that pregnancy rates are a major limiting factor. He wondered about approaches that could be used to improve pregnancy rates (e.g., multiple embryo transfer). Dr. Hanna explained that her team has attempted both single and multiple embryo transfers in rhesus macaques. Other approaches (e.g., embryo glue, surfactants) have demonstrated promise and could be applied to rhesus macaques in future studies.

Improved Methods for Freezing Marmoset Sperm
Brian Hermann, Ph.D., The University of Texas at San Antonio

Dr. Hermann spoke on improved methods for freezing sperm of common marmosets. He explained that this species is gaining popularity as an NHP model for biomedical research due to its small size, short lifespan, rapid sexual maturation, short gestation time, and production of multiple offspring per pregnancy. Dr. Hermann also noted that the marmoset can provide a more accurate recapitulation of human anatomy and physiology than rodents. The Southwest National Primate Research Center (SNPRC) receives targeted NIH funding for marmoset research.

SNPRC is interested in improving cryopreservation outcomes in marmoset sperm. Dr. Hermann noted that previous literature on this topic is sparse, with limited examples of success. His team began by defining characteristics of high-quality marmoset sperm to identify samples that are likely to yield successful results. This analysis focused on sperm morphology, semen volume, sperm count, and total motility. Additionally, the team explored approaches for enrichment of high-quality marmoset sperm. This process improved sperm motility and quality but led to decreased sperm count.

Dr. Hermann outlined the team's experimental design for cryopreservation of marmoset sperm and reported robust recovery of motile sperm. Dr. Hermann presented data demonstrating the stability of cryopreserved marmoset sperm over time. The protocol for this technique has been published in *Biology of Reproduction*. Additional experiments are in progress to assess the production of normal embryos. The team demonstrated maintained integrity of acrosome and DNA structures in cryopreserved marmoset sperm, as well as sustainment of mitochondrial activity. The ultrastructure of cryopreserved marmoset sperm also has been characterized.

Dr. Hermann detailed practical considerations for cryopreserving marmoset sperm, which include the use of store-bought eggs for test yolk buffer and liquid nitrogen vapor devices. As a next step, the team is exploring the suitability of the cryopreserved sperm for fertilization. Ultimately, these studies will provide insights into the utility of this technique for future work in the field.

Discussion

- Dr. Schmidt noted considerable variability in obtaining positive semen samples among male NHPs. Dr. Hermann agreed that this variability poses a significant challenge for his team's work. He also noted that quality can vary over time in a single NHP. The team is working on methods to improve the utility of poor-quality samples.

- Dr. Orwig inquired about IVF/ICSI success rates in marmosets, compared with rhesus macaques and other NHP models. Dr. Hermann explained that he is working within a consortium of groups focused on approaches for marmoset transgenesis. This group offers expertise in embryo editing.

- Dr. Werner Giehl Glanzner requested further details on the duration of time for assessing sperm quality. Dr. Hermann clarified that the samples were frozen for 30 minutes and thawed rapidly. They reported that some samples show improved quality within 5–10 minutes after thawing. The longer timelines, however, have not been characterized rigorously. Future studies will address this question.

- Dr. Schmidt asked about animal preparations prior to sperm collection. Dr. Hermann explained that the housing and social arrangements are not changed prior to collection. He suggested that such an approach could affect the outcomes of the collection.

Cryopreservation of Gametes and Embryos in Nonhuman Primates
Fernanda Burch, D.V.M., Ph.D., M.Sc., ONPRC

Dr. Fernanda Burch discussed cryopreservation of gametes and embryos in NHPs. She began by outlining the two-factor theory of cryopreservation, which comprises (1) intracellular ice formation and (2) solution effects. Cell injury is prevented using cryoprotective agents (CPAs), which can be permeating (i.e., small molecules that form hydrogen bonds with water and hinder crystallization) or non-permeating (i.e., large molecules that promote cellular dehydration and prevent crystallization). Cryopreservation can be achieved via slow freezing or vitrification. Dr. Burch emphasized that both the warming and cooling rates must be considered.

Slow freezing is considered the gold standard for sperm cryopreservation. These procedures have been widely studied in rhesus and cynomolgus macaques. Baboons, however, remain understudied in this area. A few studies with low success have been documented in marmosets; a new protocol from The University of Texas at San Antonio is forthcoming. Dr. Burch explained that low post-thaw longevity remains a significant issue in this area.

Vitrification is considered the gold standard for blastocysts. Dr. Burch presented an overview of NHP studies in this area, noting that rhesus macaques are the most widely studied. These studies address the use of a modified bovine fast-freezing protocol (baboons), slow freezing in a controlled-rate freezer (cynomolgus macaques), comparison of slow versus rapid freezing (cynomolgus macaques), vitrification of cleavage-stage embryos (cynomolgus macaques), vitrification with a commercial kit (rhesus macaques and marmosets), and slow freezing with dimethyl sulfoxide and glycerol (marmosets).

Dr. Burch also explained that cryopreservation of NHP oocytes has been highly challenging due to their high sensitivity compared with embryos. This greater sensitivity results from low permeability of the plasma membrane, few actin filaments in the cytoplasm, high lipidic content, vulnerability of the meiotic spindle, and early cortical granule exocytosis. Vitrification is considered the gold standard for oocytes, but no efficient protocol has been established in NHPs. Dr. Burch noted that current gaps in the field include post-thaw longevity of cryopreserved sperm, oocyte cryopreservation, and cleavage-stage embryo cryopreservation.

Discussion

- Dr. Hanna noted that some researchers have banked blastocysts. She wondered whether it would be possible to thaw, biopsy, and re-vitrify these samples. Dr. Burch agreed that such an approach is feasible and has been carried out successfully within her group. She noted that repeat biopsies also have been conducted with success.

- Dr. Hanna wondered whether it is possible to apply lessons learned across studies using Old World and New World monkeys. Dr. Burch remarked that common issues have been reported across the species groups. Furthermore, additional studies will be needed to further characterize the individual species.

- Dr. Hanna noted that squirrel monkeys and owl monkeys are available through various primate centers. She asked about the feasibility of adapting to commercial media. Dr. Burch noted that her team has used commercial media in several studies and reported results superior to those performed using homemade media. These findings suggest that the protocols are highly adaptable.

- Dr. Mary Zelinski inquired about Dr. Burch's experience using available methods for vitrification of human oocytes, with rhesus macaque oocytes used as an avenue to test that

barrier. Dr. Burch remarked that her colleagues have been pursuing methods to cryopreserve oocytes via vitrification. Methods developed for humans in the clinic were used as a starting point, but more work is needed to optimize those methods for NHPs.

Advances and Challenges of Ovarian Tissue Cryopreservation in a Nonhuman Primate Model
Alison Ting, Ph.D., Gaia Life and University of California, San Diego

Dr. Alison Ting spoke on advances in and challenges of ovarian tissue cryopreservation in an NHP model. She began by underscoring the importance of ovarian tissue cryopreservation for various applications, including fertility preservation for young female cancer patients, as well as fertility preservation for endangered species. This approach, when followed by transplantation, is the only way to reverse hormonal functions to alleviate the effects of premature menopause. This treatment is critical for maintaining cardiovascular, brain, lipid, and bone health in young female cancer survivors.

Obstacles in ovarian tissue cryopreservation include the lack of a standard protocol that demonstrates optimal ovarian function and a consistent outcome, a closed system that is safe and eliminates cross-contamination from direct contact with liquid nitrogen, and an optimized *in vivo* (i.e., transplant) or *in vitro* model that demonstrates consistent ovarian function after thawing. Dr. Ting explained that the macaque ovarian cortex displays heterogenous follicle distribution, and the follicle number decreases with age. Thus, consistency in tissue processing and collection is key to generating reproducible results.

Dr. Ting briefly outlined her team's protocol and presented data demonstrating that vitrification improves stromal and follicular morphology. She also showed their work in optimizing the composition of the vitrification solution, which was confirmed via differential scanning calorimetry. The team's temperature protocol involves slow warming (i.e., air) to avoid fracturing, followed by fast warming (i.e., water bath) to avoid devitrification.

The protocol has been validated via *in vivo* function measurements of vitrified ovarian tissue that is transplanted to heterotopic sites in rhesus macaques. Using these measurements, the team can predict the function following post-transplantation, promote the return to ovarian cyclicity, and enable the production of competent oocytes. Ultimately, the gold standard of this effort is reproduction of competent oocytes and production of normal offspring. Dr. Ting briefly outlined the heterotopic transplantation process, which has been tested in the arm (subcutaneous), abdomen (subcutaneous), omentum, and mesosalpinx. Contrast-enhanced ultrasound is used to monitor vascular development in the ovary. Dr. Ting briefly highlighted findings from these tests.

In conclusion, NHPs provide useful models for determining functional sites for transplantation of vitrified ovarian tissue. Ovarian cyclicity was restored following transplantation of vitrified ovarian tissue to heterotopic sites. More attempts at transplantation, with follow-up on fertility outcomes, are ongoing. In the future, a reproducible vitrification method for ovarian tissue will be needed for clinical applications, which will increase the availability of fertility preservation to more patients. Dr. Ting emphasized that evaluation methods take a significant amount of time, protocols can vary based on animal species and tissue size, and a balance exists between the critical warming rate and CPA toxicity.

Discussion

- In response to a question from Dr. Hanna, Dr. Ting confirmed that her group has developed shipping procedures that permit the survival of the ovarian cortex. She noted that the whole ovary can be shipped to processing sites as needed.

- Dr. Hanna asked about Dr. Ting's progress and future directions in whole-organ vitrification. Dr. Ting explained that this technique offers key advantages over other methods but is more

technically challenging to perform. She has achieved whole-organ vitrification in sheep and pig models. Oocyte collection will be the next step in this effort, and production of offspring is the ultimate goal.

Fertility Preservation in a Rhesus Model of Cancer Survivorship
Kyle Orwig, Ph.D., University of Pittsburgh

Dr. Orwig discussed cryopreservation of testicular tissues and cells in rhesus macaques. He explained that his work is focused on preserving the fertility of patients who are at risk of infertility due to various factors, including the effects of chemotherapy or radiation treatment for cancer. The current standard of care to preserve fertility in adults is cryopreservation of the patient's sperm. For patients who cannot produce a semen sample with sperm (e.g., children), a potential option is cryopreservation of immature testicular tissues, which contain spermatogonial stem cells. Dr. Orwig outlined current approaches—spermatogonial stem cell transplantation and testicular tissue grafting—for maturing those tissues and producing sperm that can be used in reproduction.

Methods for cryopreserving both cells and tissues have been established. Researchers previously compared the recovery of transplantable spermatogonia from frozen and thawed tissues with the same approach using cells. Recovery of transplantable spermatogonia was greatest in cryopreserved tissues, so these are frozen for patients. Cryopreservation of intact tissue preserves the option for tissue- or cell-based therapies in the future. This process has been used successfully to produce viable sperm, embryos, and/or offspring in animal models, suggesting that these testicular cell and tissue transplant approaches are ready for deployment in the clinic. Building on this work, the team developed protocols for testicular and ovarian tissue freezing for patients who are at risk for infertility.

Dr. Orwig presented Dr. Hermann's work in developing a monkey model for cancer survivorship. In this model, transplantation is conducted via ultrasound-guided rete testis injection. Dr. Orwig presented data demonstrating regeneration of spermatogenesis in the model. The team demonstrated that the sperm were functional and capable of fertilizing oocytes and producing embryos. More work is needed to fully demonstrate that healthy offspring can be produced using this technique.

Dr. Orwig also discussed autologous grafting of intact prepubertal testis tissue. His team transplanted immature tissues back into the animal from which they were derived. Tissues were recovered 8 to 12 months post-graft. Dr. Orwig presented an image showing tissue growth during this time. Complete spermatogenesis was observed in the sample. He also shared evidence of preimplantation embryo development after ICSI, leading to the first NHP infant born from autologously grafted, frozen, and thawed prepubertal testicular tissue. In a separate study, the team observed functional sperm in NHP tissue xenografted to a mouse host, which led to the successful birth of another NHP offspring.

Considerations for flexibility include donor sample type (e.g., testicular cells, testicular tissues, induced pluripotent stem cells [iPSCs]), cryopreservation method (e.g., controlled slow-rate freezing, commercial kits), donor age (e.g., prepubertal), recipient species (e.g., NHP, mouse), recipient age (e.g., postpubertal), and outcome (e.g., sperm, embryos, live births, transplantable stem cells).

Dr. Orwig concluded by presenting a case report of a rhesus macaque model for congenital hearing loss. He also presented a theoretical approach for model preservation and restoration by cryopreserving immature testicular tissue fragments and grafting immature tissue into postpubertal recipients. Graft tissues would be collected 8 to 12 months later, and testicular tissue would be cryopreserved with sperm in multiple aliquots. Using this tissue, oocytes would be fertilized by ICSI, and the resulting embryos can be used to establish pregnancies. Dr. Orwig noted that this approach could be used to produce a high number of male and female F1 progeny, which would allow the colony size to expand rapidly.

Discussion

- In response to a question from Dr. Zelinski, Dr. Orwig explained that vascular endothelial growth factor and hCG were administered systemically via intraperitoneal injection. He noted that multiple injections were performed over time.

- Dr. Zelinski inquired about approaches for ensuring graft success with allotransplants. Dr. Orwig confirmed that his team has completed allotransplants successfully many times. This approach allowed the team to distinguish between donor and recipient DNA. The recipient animals were treated with an immunosuppressive agent, which is costly.

- Dr. Schmidt asked about approaches to improve the extent of spermatogenesis within the grafts. Dr. Orwig explained that complete spermatogenesis was observed in nearly all the tubules. Furthermore, nearly every graft survived. He noted that collecting larger tissues has yielded better results.

- In response to a question from Dr. Julia Shaw, Dr. Orwig confirmed that his group obtained anti-CD154 from the NHP Reagent Resource. He noted that the costs have increased dramatically over the past several years.

Group Discussion
Moderators: Jenna Schmidt, Ph.D., WNPRC, and Carol Hanna, Ph.D., ONPRC

- The speakers identified immediate challenges in cryopreservation technology that must be solved first for the field to progress. It was noted that IVF/ICSI is necessary to produce offspring and is very costly. Additionally, many groups lack the infrastructure for this type of work. Competing for breeding-age females within colonies also poses a challenge.

- Other methods for long-term preservation could be considered in the future. Freeze drying could help reduce storage costs and improve accessibility for researchers.

- *In vitro* gametogenesis may offer an option for perpetuating NHP models. It was noted that this technology is likely to be feasible in the near future. Methods for maintaining iPSCs in cell culture, genetically manipulating the cells, and achieving cryopreservation are well established in NHPs and humans. More work is needed to develop a transplantable stem cell model, but the technology is advancing rapidly.

- Currently, the work toward producing oocytes is further advanced than work toward producing sperm cells. It was noted that such studies have been performed in mice and have not been translated to other species.

- Reprogramming iPSCs is more robust in humans than in NHPs. Challenges in this space include preventing the cells from differentiating, as well as maintaining karyotypes. Researchers have also encountered differences in developmental potential among iPSC lines, which drives their utility. Individual cell line variability is an additional challenge.

- Ideally, repositories could maintain the samples as both differentiated cells and stem cells. Researchers have faced challenges with reprogramming efficiency, but the additional costs associated with this effort must be considered. Cryopreserving only the fibroblasts can provide a more cost-effective option because this approach may be more straightforward and consistent across species and sexes.

- Human protocols are subject to variation across clinical sites, especially when working with tissues. The type of CPA used also may differ. Protocols must be optimized for particular tissue types.

- The community can learn lessons from differences in long-term preservation methods across NHP species. Protocols must be adapted to different species, and ramp-up development time is required.

- Meta-analyses have been performed to better characterize experimental designs for cryopreservation, but drawing firm conclusions from these studies is challenging. The variability between these parameters is significant, both among and within species. More multidisciplinary comparative analyses (e.g., multicenter studies) would be beneficial to the research community.

- The expenses associated with working with NHPs have necessitated reduced sample sizes. Additionally, ensuring reproducibility remains a challenge. Sharing protocols and fostering discussions across the community will be essential for progress in this area. These protocols are adapted over the course of years, and publications generally do not report the "final" optimized protocol. It was also noted that reproducibility-focused studies generally have lower impact factor scores than original research. *Biology of Reproduction* includes a methods section, which could be used to submit protocols.

- Future studies could involve parallel investigation of genetically modified animals and *in vitro* models—such as organoids—derived from pluripotent stem cells. It was noted that such studies would still require an animal component because these processes are too complex to model solely *in vitro* currently.

- It was noted that NHPs comprise a small but critical niche within the animal model community. iPSCs can offer potential for future preservation of these models. Rapid translation to human studies will ultimately minimize the use of animals in research studies. Cell banking will likely be a useful resource for minimizing animal studies in the future. Furthermore, the NPRC Consortium can offer the community a resource for the distribution of biomaterials.

Concluding Remarks
Bettina Buhring, Ph.D., ORIP, DPCPSI, OD, NIH

In her concluding remarks, Dr. Buhring highlighted the diversity of long-term preservation techniques and their uses across the research community. She also spoke about the complexity of cryopreservation in NHPs, noting that researchers must consider many technical variables. Dr. Buhring expressed appreciation to the attendees for their contributions and participation and adjourned the workshop.

Appendix A: Meeting Agenda

Session V. Long-Term Preservation Methods for Nonhuman Primate Models in Biomedical Research

Virtual Meeting
July 15, 2025

12:00–12:05 p.m.	**Opening Remarks** *Bettina Buhring, Ph.D., Office of Research Infrastructure Programs (ORIP), Division of Program Coordination, Planning, and Strategic Initiatives (DPCPSI), Office of the Director (OD), National Institutes of Health (NIH)*
12:05–12:45 p.m.	**Keynote Presentation: Developing New Preservation and Reanimation Methods for Gametes and Gonadal Tissues from Large Animal Models** *Pierre Comizzoli, D.V.M., Ph.D., M.Sc., Smithsonian's National Zoo and Conservation Biology Institute*
12:45–1:15 p.m.	**Species Considerations for Developing and Cryopreserving Genetic Models in Nonhuman Primates** *Jenna Schmidt, Ph.D., Wisconsin National Primate Research Center (WNPRC)*
1:15–1:45 p.m.	**Protocols and Preservation of Valuable Genetics in Rhesus Macaques** *Carol Hanna, Ph.D., Oregon National Primate Research Center (ONPRC)*
1:45–2:00 p.m.	**Break**
2:00–2:30 p.m.	**Improved Methods for Freezing Marmoset Sperm** *Brian Hermann, Ph.D., The University of Texas at San Antonio*
2:30–3:00 p.m.	**Cryopreservation of Gametes and Embryos in Nonhuman Primates** *Fernanda Burch, D.V.M., Ph.D., M.Sc., ONPRC*
3:00–3:15 p.m.	**Break**
3:15–3:45 p.m.	**Advances and Challenges of Ovarian Tissue Cryopreservation in a Nonhuman Primate Model** *Alison Ting, Ph.D., Gaia Life and University of California, San Diego*
3:45–4:15 p.m.	**Fertility Preservation in a Rhesus Model of Cancer Survivorship** *Kyle Orwig, Ph.D., University of Pittsburgh*
4:15–4:55 p.m.	**Group Discussion** *Moderators: Jenna Schmidt, Ph.D., WNPRC, and Carol Hanna, Ph.D., ONPRC*
4:55–5:00 p.m.	**Concluding Remarks** *Bettina Buhring, Ph.D., ORIP, DPCPSI, OD, NIH*
5:00 p.m.	**Adjournment**