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ABSTRACTS

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In Life
Institute of Animal Reproduction and Food Research
Polish Academy of Sciences

INTRODUCTION

The 2025 Annual Meeting of the Society for Low Temperature Biology (SLTB) was jointly hosted by the Institute of Animal Reproduction and Food Research, Polish Academy of Sciences – InLife, and the University of Warmia and Mazury, Department of Didactics and Medical Simulation.

The main focus of the SLTB2025 meeting was human fertility preservation and animal biodiversity conservation - two closely connected areas where cryobiology and low temperature technologies play a vital role. The scientific program featured invited lectures, oral and poster presentations, and dedicated sessions aimed at fostering dialogue between researchers, clinicians, and conservation specialists.

DISCREET ROLE OF DISACCHARIDES AND TEMPERATURE OF EXPOSURE IN THE HIGH EFFICIENCY OF CONTEMPORARY OOCYTE AND EMBRYO CRYOPRESERVATION

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ABSTRACT

Father Basile Luyet asked in 1973 the fundamental question: Has dehydration a cryoprotective action? Some 10 years ago, Peter Mazur's group proved that an extremely fast warming rate applied to cells vitrified after dehydration in a 0.7 molal sucrose solution allows them to survive. William Rall in 1987 speculated that partial solute permeation into cells and their simultaneous dehydration may enable successful vitrification. Papis et al. (1) proved that solutions comprising 10% glycerol and 20% propanediol, supported with 1M sucrose, vitrify and protect rabbit vitrified embryos after former saturation in the same solution devoid of sucrose. Also, Papis et al. (2) demonstrated that pre-equilibration of bovine oocytes with diluted solute (3% of ethylene glycol) at 38ºC remains harmless and that rapid dehydration applied thereafter (30 sec) in Jaffar's Ali VS14 solution allows oocytes to survive vitrification. The second role played by disaccharides in cryopreservation was their use in post-thawing/warming rehydration. Former mandatory stepwise rehydration was replaced with the straightforward use of sucrose or trehalose, allowing more efficient recovery of human embryos, human oocytes (3, 4), or even mouse embryos frozen 35 years earlier (5). Our modified approach was highly efficient for slow-frozen human oocytes. A sucrose/trehalose-driven slow-frozen oocyte rehydration greatly enhances the survival rate and clinical effects (6). Jürgen Liebermann, following his recent successful fast warming method, utilizing 1 or 0.5 molar sucrose solution, has suggested that too high sucrose concentration may prevent rapid rehydration. Our observations support the notion that excessive shrinking of oocytes after thawing (or warming) may become detrimental. In conclusion, even perfect ideas sometimes need 50 years to be confirmed and come through. And, that some not-so-obvious factors (such as disaccharides in cryopreservation) may become helpful or even indispensable if the general conditions change accordingly.

REFERENCES

1. Papis K, Fujikawa S, Kojima T & Oguri N (1993) *Cryobiology* **30**, 98–105 https://doi.org/10.1006/cryo.1993.1009
2. Papis K, Shimizu M & Izaike, Y (2000) *Theriogenology* **54**, 651–658 https://doi.org/10.1016/S0093-691X(00)00380-0
3. Parmegiani L, Tatone C, Cognigni GE et al (2014) *Reproductive Biomedicine Online* **28**, 614–623 https://doi.org/10.1016/j.rbmo.2014.01.015
4. Papis K, Lewandowski P, Stachowiak E et al (2015) *Human Reproduction* **30**, 220-221.
5. Papis K, Grzesik H, Pytel A, Niwińska A, Gajewski Z (2022) in *19th Int. Cong. Animal Reprod. Bologna, Proceedings*, **M151**, 93.
6. Papis K, Hardej K, Stachowiak E et al (2025) *Reproductive Biology and Endocrinology* **23**, 58. https://doi.org/10.1186/s12958-025-01383-2

SOFT SHIELDS FOR DELICATE TISSUES: ENGINEERED ALGINATE HYDROGELS IMPROVE OVARIAN CRYOPRESERVATION OUTCOMES

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ABSTRACT

Cryopreservation of ovarian tissue is a key strategy for fertility preservation, yet it often leads to structural damage and loss of viability. This study aimed to develop and evaluate an optimised alginate-based hydrogel to improve ovarian tissue protection during cryopreservation. Using Response Surface Methodology, we optimised an alginate hydrogel formulation by tuning calcium carbonate ($CaCO_3$) and D-glucono-δ-lactone (GDL) concentrations to achieve desirable viscoelastic properties. This optimised hydrogel was then applied to encapsulate lamb ovarian cortex, which was subsequently divided into three groups: (I) fresh control, (II) non-encapsulated frozen (Frozen), and (III) alginate-encapsulated frozen tissue (Frozen-gel). Tissues were frozen, thawed, and cultured in vitro for 24 hours. Follicular integrity, stromal cell density, apoptosis (caspase-3), vascularisation (CD31, VEGF), extracellular matrix preservation (collagen IV), and fibrosis were assessed via histology and immunostaining. The optimised hydrogel (0.98% alginate, 52 mM $CaCO_3$, 107 mM GDL) closely mimicked the viscoelastic properties of native ovarian tissue. The Frozen-gel group showed significantly improved follicular morphology and stromal cell density compared to non-encapsulated frozen tissue. VEGF and CD31 expression levels were partially preserved, while collagen IV levels were maintained. Caspase-3 expression and fibrotic remodeling were reduced, though not significantly. These findings highlight the potential of optimised alginate hydrogels to improve cryoprotection by preserving ovarian tissue structure, reducing apoptosis, minimising fibrosis, and supporting vascular and extracellular matrix integrity, offering a promising strategy for ovarian tissue preservation.

CUMULUS CELL RECONSTRUCTION RESCUES MATURATION POTENTIAL IN VITRIFIED IMMATURE OOCYTES

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ABSTRACT

Cryopreservation of immature cumulus-oocyte complexes (COCs) compromises their subsequent in vitro maturation (IVM), limiting their use in fertility preservation programs (1). This may result from the disruption of transzonal projections (TZPs), which mediate communication between oocytes and cumulus cells (CCs) and are crucial for oocyte competence. This study aimed to evaluate TZP integrity and lipid droplet (LD) distribution in cryopreserved immature bovine oocytes matured in various culture systems. Immature COCs were collected from a local abattoir and randomly assigned to four IVM groups: 1 intact COCs, 2 denuded oocytes (DOs), 3 DOs co-cultured with CCs in a simple drop system, and 4 DOs with CCs in a two-step COC reconstruction system. Oocytes and COCs were vitrified using the Cryotop method, while CCs were cryopreserved by slow freezing (2). Our findings showed no significant change in LD number, diameter, or occupied area in DOs after IVM. In contrast, oocytes matured in intact COCs exhibited increased LD parameters. The addition of CCs to DOs, either in a drop culture (group 3) or via the COC reconstruction system (group 4), significantly enhanced LD number, diameter, and total LD area. TZPs were nearly absent in DOs and only partially restored in group 3. However, the two-step reconstruction system led to an initial increase in TZP number, followed by a decrease mimicking natural COC maturation dynamics. Cryopreservation of COCs reduced both LD content and TZP number. Notably, the reconstructed COC system restored these parameters in cryopreserved oocytes to levels comparable to those observed in fresh, intact COCs after IVM. We conclude that CCs play a critical role in LD accumulation during final oocyte maturation and that vitrification disrupts this process by impairing CC–oocyte connections. The proposed two-step COC reconstruction system offers a promising strategy to restore functional interactions and lipid accumulation in cryopreserved immature oocytes.
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REFERENCES

1. Brambillasca F, Guglielmo MC, Coticchio G, Mignini Renzini M, Dal Canto M & Fadini R (2013) *J Assist Reprod Genet* **30**,1531-1539. https://doi.org/10.1007/s10815-013-0112-0
2. Yurchuk T, Likszo P, Witek K, Petrushko M & Skarzynski DJ (2024) *Int J Mol Sci*. **25,** 6074. https://doi.org/10.3390/ijms25116074

COLD PRECONDITIONING PRIOR TO OVARIAN TISSUE CRYOPRESERVATION

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ABSTRACT

For girls undergoing gonadotoxic cancer treatments, ovarian tissue preservation is the only option to preserve fertility. Oocyte extraction is not possible in children. Many countries adopt a hub-and-spoke

model where ovarian tissue is removed and cryopreserved at a central site. This can present practical challenges and is a potential source for variation between samples. While time outside the body unfrozen is generally considered detrimental, it has been shown that for ovarian tissues, a period of 24 to 48h where the tissue chilled to 4°C before cryopreservation improves key tissue assessment parameters post-thaw. This study investigated this pre-chilling, using TOM20 (translocase at the outer membrane of the mitochondria) as a biomarker of mitochondrial metabolism to assess oxidative phosphorylation and HSP70 as a marker of oxidative stress. Ovaries from 19d-old mice (F1:C57B/6xCD1) were immediately fixed (0h) or stored in transport media at 4°C for 12h, 24h, 36h, or 48h before fixation. Granulosa cells in secondary follicles showed reduced TOM20 levels after 24h (32.15±11.91, n=21) 36h (33.38±10.72, n=15), and 48h (32.52±15.83, n=17) of cooling compared to immediately fixed tissues (0h: 56.54±11.97, n=19; $P<0.001$), but TOM20 expression remained constant at the timepoints measured in oocytes. HSP70 showed that ovarian cells experienced increased stress, with expression peaking at 36h at 4°C in oocytes and granulosa cells. This presents the intriguing possibility that limited oxidative stress on ovarian tissue may result in increased cryopreservation success, perhaps by activating cells' anti-oxidant pathways before the greater stress of cryopreservation. The effects of cold on multicellular systems in nature has been well reported, from increases in polyunsaturated fat content of cell membranes, reduced metabolism, increased oxygen consumption and oxidative stress, and angiogenesis. This study begins the process of isolating the impact of each effect on ovarian tissue, which ultimately could be used to improve fertility outcomes in girls with cancer.

COOLED OVERNIGHT TRANSPORT IN HISTIDINE-TRYPTOPHAN-KETOGLUTARATE SOLUTION MODULATES APOPTOSIS AND MITOCHONDRIAL PROTEINS IN OVARIAN TISSUE PRIOR TO CRYOPRESERVATION

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ABSTRACT

As the number of clinics without in-house cryopreservation facilities for ovarian tissue increases, overnight transport (ONT) is gaining importance for the safe and efficient use centralized of centralized cryobanks. Previous studies have shown that ovarian tissue remains viable for up to 24 hours under controlled transport conditions (1-5). However, the molecular effects of ONT using Histidine-Tryptophan-Ketoglutarate (HTK) solution—particularly on mitochondrial function, oxidative stress, and apoptosis—are not yet fully understood. This ongoing pilot study investigates molecular changes in ovarian tissue following ONT in HTK solution at 4–8 °C, focusing on protein markers related to apoptosis, oxidative stress, and mitochondrial integrity. Ovarian tissue from 12 breast cancer patients (mean age: 30.5 years; mean AMH: 2.75 ng/ml) was collected with ethical approval and informed consent. Each sample was divided into: Group 1 (Fresh): Processed Protein expression was assessed via immunohistochemistry and/or Western blot for apoptosis: Caspase-3, BAX/BCL-2, Cytochrome C and mitochondria: TOM20, COX IV, SOD1 Some ONT samples showed higher BCL-2 expression and stable or elevated levels of TOM20 and COX IV compared to fresh samples, suggesting reduced apoptotic signaling and preserved mitochondrial integrity. These findings point to a protective effect of cooled HTK during transport. ONT in cooled HTK appears to preserve mitochondrial function and limit apoptosis, potentially improving tissue quality prior to cryopreservation. Our results support ONT as a safe, practical solution for clinics without local cryobank, helping ensure the viability of patient samples during transport. Further research with larger cohorts is necessary to confirm and optimize this approach.

ABANDONED EMBRYOS DILEMMA: HOW LONG-STORED FROZEN EMBRYOS CAN SUPPORT AN EMBRYO DONATION PROGRAM – THE NOVUM CLINIC PERSPECTIVE

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ABSTRACT

The storage of surplus frozen embryos, which may be considered "abandoned" after the biological parents achieve successful parenthood, has sparked extensive discussion regarding the legal and ethical implications of embryo disposition. Under Polish law, embryos must be stored indefinitely unless donation becomes possible. Numerous scientific studies have confirmed the long-term safety of frozen embryo storage, including reports on viable mouse embryos after 35 years of storage (1) and successful human pregnancies and births after 20 years (2, 3). This study aimed to evaluate the impact of the cryopreservation method (slow freezing using DMSO or propanediol versus vitrification) and storage duration (ranging from 1 to 19 years) on the clinical outcomes of the embryo donation program run by the nOvum Clinic. We retrospectively analyzed 366 embryo donation cases accomplished between 2019 and 2022. Of 684 embryos thawed, 69.01% survived and were deemed suitable for transfer. In total, 342 frozen embryo transfers (FETs) were conducted, resulting in 142 biochemical pregnancies (41.52% per FET), 124 clinical pregnancies (36.26%), 96 deliveries (28.07%), and 100 live births (29.24%). No statistically significant differences in pregnancy or birth rates were observed regarding the cryopreservation method or storage duration ($P > 0.05$). However, vitrification resulted in a significantly higher post-thaw embryo survival rate (83.33%) compared to the slow-freezing method using propanediol (59.29%, $P \leq 0.05$).The nOvum Clinic donation program, established 20 years ago, has given birth to 243 babies. The data presented here confirm satisfactory outcomes, regardless of cryopreservation method and storage time duration, and prove rational embryo disposition. Babies born due to this can be treated as a surplus benefit of our approach.

REFERENCES

1. Papis K, Grzesik H, Pytel A, Niwińska A & Gajewski Z (2022) in *19th Int. Congress on Animal Reprod. Bologna, Proceedings*, **M151**, 93
2. nOvum 2025, unpublished.
3. Dowling-Lacey D, Mayer JF, Jones E, Bocca S, Stadtmauer L & Oehninger S (2011) *Fertil Steril* **95**, 1120.e1-1120.e11203. https://doi.org/10.1016/j.fertnstert.2010.08.056

CRYOPRESERVATION OF THREE-DIMENSIONAL CELL SPHEROIDS

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ABSTRACT

Three-dimensional (3D) spheroid cultures hold promise for regenerative medicine, disease modelling, and drug discovery. These systems mimic in vivo tissue architecture, enabling more accurate cellular responses and enhancing regenerative potential. Cryopreservation could support their widespread use, but its impact on spheroid viability and function remains underexplored. In this study, we evaluated the functional properties of 3D spheroids formed from human multipotent mesenchymal stromal cells (MSCs) and glioblastoma (GBM) cell lines, along with their responses to classical slow-rate cryopreservation. MSC spheroids were characterized for paracrine activity, and metabolic landscape, while GBM spheroid growth and invasion were analysed using an Incucyte-based assay. Mixed MSC–GBM spheroids (1:1 ratio) were also examined for effects on GBM behaviour and post-cryopreservation viability. Cryopreservation was performed with 10% Me_2SO and 20% FBS, using the cooling rate 1°C/min to –80°C, then stored at –196°C for one week before thawing. Cell viability was assessed by Live/Dead staining and Alamar Blue assays. GBM invasion was further studied in a 3D collagen hydrogel. MSC spheroids displayed enhanced paracrine secretion and a metabolic shift, with remodelled metabolomic and lipidomic profiles. Cryopreservation caused a 20–30% viability loss. GBM spheroids showed cell line–dependent differences in growth, architecture, invasion, and cryopreservation response, though post-thaw viability remained above 55%. Mixed MSC–GBM cultures altered growth kinetics of aggregates but did not significantly affect cryopreservation outcomes. Therefore, we provide a functional characterization of MSC and GBM spheroids and assess cryopreservation effects on their viability. Standard protocols caused moderate but non-critical viability loss, supporting further optimization for complex 3D culture systems.

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CRYOPRESERVATION OF AT-MSC IN 3D BIOENGINEERED CONSTRUCTS

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ABSTRACT

Cryopreservation of three-dimensional (3D) bioengineered constructs presents significant challenges in regenerative medicine and tissue engineering. The success of cryopreservation depends on several factors, including the type of cells used, the properties of the extracellular matrix (ECM), density and architecture of the 3D construct. This study aimed to establish various types of 3D cultures based on multipotent mesenchymal stromal cells (MSCs), with or without ECM hydrogels, and to assess the effects of cryopreservation on cell viability and metabolic activity. Human adipose tissue-derived MSCs were cultured for 3 and 7 days under different 3D conditions: (a) ECM-free spheroid cultures and (b) ECM-supported hydrogel cultures (Collagen I and blood plasma-based hydrogels). The cryoprotective medium consisted of 10% DMSO and 10% FBS in alpha-MEM. Constructs were cryopreserved at a cooling rate of 1°C/min down to -80°C, followed by storage at -196°C for a minimum of 7 days. Metabolic activity and viability were assessed 24 hours post-thaw using the Alamar Blue and LIVE/DEAD assays,

respectively. Distinct 3D culture environments resulted in noticeable differences in MSC morphology, metabolic activity, gene expression, and secretome profiles, which varied according to the biomaterial type and culture duration. Cryopreservation efficiency was higher in 7-day cultures (60–80%) compared to 3-day cultures (40–55%). Additionally, cryopreservation outcomes varied depending on the type of 3D construct. In conclusion, the overall architecture, maturation stage, and type of the bioengineered construct significantly influence cryopreservation efficacy. These factors should be carefully considered when optimizing biopreservation strategies for 3D grafts in regenerative medicine applications.

INVESTIGATION OF CORE-SHELL GELATIN/ALGINATE CAPSULES FOR MSCS ENCAPSULATION AND TRANSPORTATION

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ABSTRACT

Encapsulation of mesenchymal stem cells (MSCs) offers a valuable strategy in regenerative medicine and tissue engineering, facilitating improved cell viability, function, and delivery. Using gelatin/alginate core-shell capsules creates a protective niche that supports MSC growth and structural integrity. This technique is an effective alternative to cryopreservation and costly infrastructure, allowing safer, simpler transport of MSCs at ambient temperature while maintaining therapeutic potential. This work explores optimization of encapsulation conditions and investigates MSC behavior within these microenvironments. The goal is to encapsulate human MSCs in gelatin/alginate core-shell capsules, optimize process parameters for consistent capsule quality, and assess viability, metabolic activity, and spatial organization of encapsulated cells. Emphasis is placed on how gelatin concentration affects MSC behavior at physiological and room temperatures. Capsules were fabricated by coaxial electrospraying using gelatin concentrations from 2.5% to 12.5% (w/v) and varying core-to-shell flow rate ratios. Viability was assessed via FDA/EthD staining and metabolic activity with Resazurin assay. Capsule morphology and rheological properties were analyzed to link physical structure with biological performance. Gelatin concentrations of 2.5%–7.5% with flow ratios 1:4–1:8 yielded capsules of optimal stability and uniformity. MSCs retained fibroblast-like morphology and showed increased viability and metabolic activity over 17 days in culture. Encapsulation significantly reduced stress-induced apoptosis compared to monolayer culture. Gelatin/alginate encapsulation supports MSC viability and function, offering a scalable solution for cell therapy delivery. This study demonstrates how tuning encapsulation parameters enhances cell quality for translational applications. Next, we will investigate long-term MSC functionality post-encapsulation, assess differentiation potential, and develop protocols for large-scale capsule production following international quality standards for clinical applications.

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CRYOPRESERVATION OF PORCINE BLOOD BY DROPLET-BASED VITRIFICATION METHODS

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ABSTRACT

Storage of blood donations requires addition of anticoagulants and stabilising substances immediately after collection. The shelf life of whole blood of max. 21 days at 2-6 °C can be extended by centrifugation to obtain red blood cell concentrates, which can be stored for up to 49 days. At the end of this period, unused blood bags must be disposed - in Germany around 800 units per day. A longer shelf life via cryopreservation would therefore be desirable. Red blood cells require very high cooling rates of about 3500 K/min and high amounts of cryo-protectants (CPA) such as glycerol of up to 40 % (w/v). Inhomogeneous temperature and CPA distributions in the 500ml blood bags are problematic. Thus, we investigate a new approach of droplet-based vitrification processes for porcine whole blood in order to enable very high cooling and thawing rates. We compare porcine blood microdroplets realized via microfluidic and air flow methods. The improved surface-to-volume ratio had a positive effect on the achievable cooling rates and the more homogeneous temperature distribution in the droplets. So far, with the air flow method the microdroplets have diameters below 250 µm with leads to surface to volume ratios above 20.000 as compared to blood bags with less than 100 m2/m3. Cooling rate of up to 1000 K/min as compared to blood bags of 240 K/min via HES rapid cooling were achieved. By varying the thawing protocol, the rate of hemolysis could be further reduced in all procedures. Next steps include further reducing the rate of hemolysis and increasing the thawing rate.

CRYOPRESERVATION OF UNRELATED HAEMATOPOIETIC CELL ALLOGRAFTS – A SINGLE CENTRE EXPERIENCE

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ABSTRACT

The COVID-19 Pandemic led to worldwide application of haematopoietic progenitor cell (HPC) cryopreservation to increase HPC allotransplantation safety. In this study we evaluate the quality of unrelated HPC allografts transplanted either as cryopreserved or fresh in the University Hospital Hradec Králové in the years 2020, 2021. Unrelated HPC allograft cryopreservation according to the institutional protocol (1) was done in 19 cases and in 20 cases allografts were transplanted as fresh. Similar indications (acute and chronic leukaemia, aplastic anaemia, myelodysplastic syndrome, myelofibrosis and malignant lymphoma) were encountered in both groups and differences in age, sex and diagnosis were insignificant ($p = 0.79, 0.75, 0.25$). HPC allografts were collected mostly in licensed European Union Apheresis Collection Facilities. The quality of cryopreserved grafts (white blood cell count, CD 34+ cell count, post – thaw nucleated (NC) and mononuclear cell viability and cell potency) was assessed from thawed control

samples. Cell viability and in case of cryopreserved grafts also cell potency tests were repeated at infusion. The clinical outcome was assessed by evaluation of neutrophil and platelet engraftment times. Statistical comparison was made by non-parametric Mann-Whitney test at $\alpha = 0.05$. All cryopreserved grafts were released for transplantation, one graft remained unused for clinical reasons. All 20 fresh grafts were transplanted. There were no significant differences in infused HPC doses: median viable NC/kg 6.5 x 108, median CD34+ cell/kg 7.32 x 106 in the cryopreserved graft group (CGG) and median NC /kg 7.18 x 108 and median CD34+cell/kg 5.76 x 106 in the fresh graft group (FGG) (p = 0.12, 0.12). The post thaw HPC potency using CFU-GM (colony forming unit–granulocyte–macrophage) testing was well preserved (median CFU-GM/kg 7.81x 105). There was a statistically insignificant prolongation of neutrophil engraftment time: Median16.5 days in the CGG, 13 days in the FGG (p= 0.33) while median platelet engraftment times were practically identical: 13 days in the CGG, 14 days in the FGG (p= 0.87).

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REFERENCES

1. Mericka P, Jandova M, Fuller B et al (2024) *Probl. Cryobiol. Cryomed.* **34**, 71-112 https://doi.org/10.15407/cryo34.02.071

MICROBIOLOGICAL FINDINGS IN CRYOCONTAINERS AND DEEP-FREEZE BOXES: ANALYSIS OF FINDINGS FROM FREEZING AND THAWING WITH EMPHASIS ON THE SOURCE OF CONTAMINATION

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ABSTRACT

Cryogenic containers are used for long-term storage of biological materials such as cells and tissues, but may represent an overlooked source of contamination due to condensation, frequent opening, and improper handling. The aim of this study was to perform microbiological analysis using standard cultivation techniques with evaluation using mass spectrometry MALDI-TOF: (1) from the interior of cryocontainers operating on the basis of liquid nitrogen vapor ($T \leq -150$ °C) including samples of frost from the lid and wall using microbiological swabs, and samples of liquid from melting ice from the bottom of the device using quantitative measurement, (2) from the interior of deep-freezing boxes ($T \leq -80$ °C) using microbiological swabs or quantitative measurement, with a focus on the potential risk of spreading microorganisms through liquid nitrogen vapor (3). The results of quantitative microbiological examination in 8 cryocontainers revealed microbial growth 2-10^2 CFU/mL in 5 cryocontainers. Potentially pathogenic microorganisms such as *Delftia tsuruhatensis*, *Acinetobacter lwoffii*, *Acinetobacter pittii*, *Chryseobacterium* species, *Pseudomonas monteilii*, *Moraxella osloensis*, *Pseudomonas koreensis* and *Pseudomonas oryzihabitans*, were isolated; pathogenic microorganisms such as *Staphylococcus aureus*, and non-pathogenic bacteria such as *Micrococcus species*, *Kocuria kristinae*, and P*aenibacillus amylolyticus* were found. Microbiological swab results from the inner wall of 12 cryocontainers showed the presence of microorganisms such as Bacillus species, *Acinetobacter pittii*, *Acinetobacter lactucae*, *Aerococcus viridans*, and *Pseudomonas oryzihabitans*. All results of swabs taken from the lid of 9 cryocontainers were culture negative. Microbiological swabs taken from the inside of 6 deep-freeze boxes

revealed the presence of *Acinetobacter pittii*, coagulase negative staphylococci and *Pseudomonas oryzihabitans*. Quantitative testing of 5 icing samples then revealed low contamination ranging from 1 to 14 CFU/mL and included the following findings: *Bacillus muralis, Bacillus species, Bacillus simplex, Acinetobacter pittii, Micrococcus* species, and coagulase negative staphylococcus. Swabs taken from protective gloves showed the same contamination as in both types of freezing devices.

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REFERENCES

1. Morris GJ (2005) *Cryobiology* **50**, 231-8.
2. Grout BW & Morris GJ (2009) *Theriogenology* **71**, 1079-82 https://doi.org10.1016/j.theriogenology.2008.12.011
3. Bajerski F, Bürger A, Glasmacher B, Keller ERJ, Müller K, Mühldorfer K, Nagel M, Rüdel H, Müller T, Schenkel J & Overmann J (2020) *Appl Microbiol Biotechnol* **104**,131-144 https://doi.org/10.1007/s00253-019-10242-1

ADULT, NO IMPRINTING, MEMORY IS RETAINED AFTER VITRIFICATION OF *C. ELEGANS*; A MODEL OF HUMAN MEMORY CRYOPRESERVATION: ANSWER TO CRITICISMS

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ABSTRACT

Cryopreservation has been extensively explored as a means to enable the long-term storage of biological systems, with implications spanning fields from healthcare to space travel. A key challenge remains in ensuring that intricate biological processes, such as memory retention, are preserved after vitrification and reanimation. This work addresses a major criticism of a prior study by Vita-More and Barranco (1), which reported preservation of imprinting memory in *C. elegans* cryopreserved at the L1–L2 larval stage. Critics argued that imprinting, although a valid form of memory in nematodes, is not present in adult humans and thus less relevant to the goals of human cryopreservation. In contrast, our study uses adult worms and demonstrates for the first time the preservation of a memory type analogous to that found in adult humans. The research followed a two-step approach. Initially, adult C. elegans were trained to associate the odorant butanone with the presence of food, forming a basic associative learning. Following conditioning, the worms were cryopreserved using the SafeSpeed technique (2), which utilizes capillaries to achieve extremely fast cooling and warming rates. Once reanimated, the nematodes underwent a chemotaxis assay to assess memory retention. Control groups consisted of trained animals that were not subjected to vitrification. The data demonstrated that vitrified adult worms retained the conditioned association between butanone and food after cryogenic exposure and revival. Their memory index did not significantly differ from that of the non-vitrified trained controls, indicating that neural circuits involved in encoding memory remained operational. These findings strengthen the case for the potential of cryopreservation to maintain cognitive-like functions and emphasize the utility of *C. elegans* as a model for exploring the boundaries of memory preservation in biological systems. The effective use of a high-speed vitrification method to sustain memory integrity suggests promising avenues for advanced applications, including organ preservation and future biostasis in humans. Moreover, this work enhances our understanding of memory durability in simple organisms, serving as a foundation for further studies into long-term memory, neural robustness, and the prospects of deep cryogenic storage for complex life.

REFERENCES

1. Vita-More N, Barranco D. Persistence of Long-Term Memory in Vitrified and Revived Caenorhabditis elegans. Rejuvenation Res. 2015 Oct;18(5):458-63. doi: 10.1089/rej.2014.1636. Epub 2015 Aug 20. PMID: 25867710; PMCID: PMC4620520.
2. Barranco D, Cabo-Ruiz V, Risco R. Use of fine capillaries for cryopreservation of Caenorhabditis elegans by vitrification. Cryobiology. 2023 Dec;113:104585. doi: 10.1016/j.cryobiol.2023.104585. Epub 2023 Sep 9. PMID: 37690502.

WARMING WITH FOCUSED ULTRASOUND: FROM KERNELS TO ORGANS

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ABSTRACT

The warming of cryopreserved samples poses various challenges. The presence or absence of cryoprotectants determines specific requirements in warming speeds. Numerous cryoprotecting solutions exist to achieve sufficiently high warming rates with current technologies. However, as sample size increases, attaining uniform and adequate warming becomes increasingly difficult. Thermal gradients between warmer and colder regions can lead to fracturing. While mild-warm water baths are commonly used, they are suboptimal for large samples. Boundary warming promotes temperature gradients, risking internal recrystallization and surface overheating. Thus, warming must not only be fast and uniform but also penetrate deeply. Several technologies have emerged to address these challenges. Nanowarming has proven effective in thawing small hearts and kidneys (1). Dielectric warming offers high warming rates in tens of millilitres, and both methods provide fast, penetrating, and relatively homogeneous heating (2). Recently, High-Intensity Focused Ultrasound (HIFU) has gained attention as another promising approach (3-5). Effective rewarming with HIFU requires precise control of the acoustic field and its conversion into thermal energy. Achieving homogeneous, high-rate warming in large volumes is still in early development. Due to the complexity of nonlinear acoustic propagation, a hybrid in silico and in vitro methodology is essential. We present our latest advances in warming small organs at high rates and examine scalability using a 128-phased array. COMSOL Multiphysics simulations were used to assess the effects of multi-focal warming. Experimentally, we employed a 600 W HIFU system to generate warming volumes suitable for small organs inside cryovials. From a focal size of 2 x 7 mm, we managed to scale this volume to warm a small organ-like phantom. Combining simulations and experiments, we achieved warming rates exceeding 200 °C/min in multi-focal configurations for samples stored at -120 °C, and we explored strategies to scale up warming volumes while maintaining or increasing warming rates.

REFERENCES

1. Han Z, Rao JS, Gangwar L, Namsrai BE, Pasek-Allen JL, Etheridge ML, Wolf SM, Pruett TL, Bischof JC & Finger EB (2023) *Nat Commun* **14**, 3407 https://doi.org/10.1038/s41467-023-38824-8
2. Wowk B, Ting A, Phan J, Pagotan R, Sharif A, Chow L & Fahy GM (2025) *Cryobiology* **119**,105257 https://doi.org/10.1016/j.cryobiol.2025.105257
3. Alcalá E, Encabo L, Barroso F, Puentes A, Risco I & Risco (2023) *R. Sci Rep* **13**, 7603 https://doi.org/10.1038/s41598-023-34681-z
4. Encabo L, Alcalá E, Lopez-Soria J, Barroso F, Gonzalez-Suero C, Jimenez JJ, Armas V, Risco I & Risco R (2024) *Transplantation* (2024) **108**, e15-e17.

https://doi.org/10.1097/TP.0000000000004846

5. Xu R, Treeby BE & Martin E. J. (2023) *Acoust. Soc. Am* **153,** 517–528. https://doi.org/10.1121/10.0016886

COMPARATIVE STUDY OF CRYOPROTECTANT LOADING IN MOUSE HEART, MOUSE KIDNEY, AND SHEEP OVARY USING X-RAY COMPUTED TOMOGRAPHY

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ABSTRACT

The controlled delivery of cryoprotectant agents (CPAs) is a critical step in whole-organ cryopreservation, where toxicity and heterogeneous distribution can severely compromise tissue viability. To address this, we conducted a comparative study of CPA (VMP solution) loading in three different organ types: mouse heart, mouse kidney, and sheep ovary—the latter being a widely accepted preclinical model for human ovarian tissue. Each organ was excised, cannulated, and connected to a perfusion circuit adapted to its anatomical characteristics. The loading process was monitored in real time using X-ray Computed Tomography (CT), which enabled non-invasive tracking of CPA penetration. Me_2SO, a major component of the VMP solution, provided strong X-ray attenuation, making it suitable for quantitative imaging. The imaging protocol included calibration of the CT system (Bioscan nanoSPECT/CT®, Mediso, Hungary) to detect Me_2SO concentrations in the 0–50% range, with scans performed at 65 kVp, 1500 ms exposure per projection, and 100 µm resolution. Acquisition timepoints were chosen to capture the progression of CPA infiltration across organ regions. Image reconstruction was carried out using VivoQuant (Invicro, USA) and analysed with PMOD 4.3 (PMOD Technologies LLC, Switzerland). Our comparative analysis revealed distinct patterns of CPA distribution in the three organs, reflecting differences in vascular architecture and permeability. These findings offer essential insights into organ-specific loading kinetics, with direct implications for the development of optimised vitrification protocols tailored to each tissue type. This work establishes a foundational reference for future studies on cryopreservation in complex organs and supports ongoing efforts to implement advanced rewarming technologies.

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ADVANCING REWARMING TECHNOLOGIES IN CRYOPRESERVATION: HIGH-INTENSITY FOCUSED ULTRASOUND FROM FOUNDATIONAL STUDIES TO WHOLE-ORGAN APPLICATIONS

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ABSTRACT

The cryopreservation and subsequent rewarming of biological tissues and organs remain among the most significant technical barriers in transplantation and biobanking. Traditional rewarming techniques often lead to deleterious effects such as thermal gradients, recrystallization, and fracturing. In this context, High-Intensity Focused Ultrasound (HIFU) is gaining prominence as a transformative, non-invasive technology capable of delivering fast and spatially uniform heat while remaining compatible with cryoprotectant agents. This presentation offers a comprehensive synthesis of our multi-stage research programme on HIFU-mediated rewarming. Early experimental efforts validated the use of HIFU for preventing recrystallization in cryopreserved C. elegans, and for mitigating second warm ischemia prior to anastomosis in murine hearts. These studies demonstrated superior morphological preservation and functional outcomes relative to conventional methods. Complementary finite-element simulations supported these results by confirming the system's ability to deliver warming rates above 100 °C/min with minimal spatial thermal gradients, thus fulfilling the physical requirements to avoid devitrification and ice recrystallization. Recent work has extended the application of HIFU to more structurally complex tissues. Aortic segments cryopreserved at –80 °C were successfully rewarmed using HIFU, yielding enhanced cellular viability and absence of macro- or microfractures when compared to standard water bath warming. Moreover, we contextualize HIFU within the broader landscape of rewarming technologies. Nanowarming offers excellent homogeneity via dispersed magnetic nanoparticles, while dielectric heating delivers volumetric warming through electromagnetic fields. HIFU complements these by offering a clean, contact-free strategy with the added benefit of real-time feedback and control using MRI or acoustic interferometry. As a novel contribution, we present new experimental data demonstrating the successful application of HIFU for the rewarming of vitrified whole mouse kidneys and hearts. Without attempting post-warming functional recovery, we confirmed structurally homogeneous rewarming with rates exceeding 100 °C/min in both organs, reinforcing the feasibility of HIFU for future organ-scale cryopreservation protocols. By integrating historical insights with new organ-level data, this work highlights the evolution of HIFU from proof-of-concept models to full-organ systems. These advances solidify its role as a central technique in next-generation biopreservation platforms, paving the way for robust cryobanks and improved clinical outcomes in transplantation.

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CRYOGENIC CONTRAST-ENHANCED MICROCT: A NON-DESTRUCTIVE 3D IMAGING TOOL TO ASSESS EFFECT OF FREEZING ON TISSUE MICROSTRUCTURE

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ABSTRACT

Cryopreservation is a cornerstone of modern biobanking, fertility preservation, and regenerative medicine. Yet, evaluating the structural integrity of tissues post-freezing remains a major challenge. Classical 2D histology (involving tissue embedding, slicing, staining and microscopy evaluation) is, while widely used, inherently destructive and limited in its ability to capture the 3D microstructure of tissues (1). To address this, we present cryogenic contrast-enhanced microfocus X-ray computed tomography (cryo-CECT), a nondestructive imaging modality that enables high-resolution 3D visualization of soft tissue microstructure in the frozen state (2). Cryo-CECT combines microCT with contrast-enhancing staining agents (CESAs), leveraging the phase segregation between CESAs and water during freezing to enhance contrast between

tissue constituents. It allows direct assessment of freezing-induced alterations in tissue microstructure, offering a unique tool for evaluating and optimizing cryopreservation protocols. Recent work by Maes et al. (2) demonstrated the power of cryo-CECT to visualize the effect of the freezing rate and temperature on individual muscle and collagen fibers of the heart and tendon, respectively, while a follow-up study (3) applied cryo-CECT to assess the impact of freezing rate on kidney microstructure. This study revealed that freezing protocols significantly influence tissue integrity, with slower rates causing deformation and faster rates preserving fine structures. These findings underscore the relevance of cryo-CECT in refining cryopreservation strategies. We further illustrate the utility of cryo-CECT in ovarian tissue, where it enables volumetric follicle staging and assessment of extracellular matrix organization—key indicators of tissue viability post-thaw. By correlating cryo-CECT with classical 2D histology, we validated its sensitivity to cryo-damage and remodelling. Cryo-CECT thus emerges as a powerful, nondestructive tool for 3D structural assessment of cryopreserved tissues, bridging the gap between imaging and preservation science, and opening new avenues for quality control and protocol development in cryobiology.

REFERENCES

1. Hoffmann D et al (2025) *Microsc. Microanal* **31**, https://doi.org/10.1093/mam/ozaf048.1094
2. Maes A et al (2022) *Nat Commun* **13** , 6207 https://doi.org/10.1038/s41467-022-34048-4
3. Maes, A. et al (2024) *Int J Biomed Imaging* **2024**, *3924036* https://doi.org/10.1155/2024/3924036

MODULAR SEMI-PASSIVE COOLING DEVICE FOR CONTROLLED CRYOPRESERVATION OF BIOLOGICAL SAMPLES

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ABSTRACT

Cryopreservation is essential for preserving the quality and extending the shelf life of biological materials such as red blood cell concentrates. When dealing with cell suspensions, slow freezing remains the most widely used technique. Various technical solutions have been developed for such applications, generally falling into two categories: active cooling systems (e.g. those using vaporized liquid nitrogen) and passive cooling methods (e.g. relying on insulation in a -80 °C freezer). While active systems offer greater control over the cooling process, they typically involve high cost, specialized equipment, and are dependent on external coolant sources. In this work, we present a prototype of a modular, semi-passive cooling device developed to enable controlled and reproducible cooling of biological samples. The system regulates heat transfer between a thermally conductive sample holder and pre-cooled, high heat capacity cells through an actively controlled insulating layer. Thanks to its modular design, the device can be adapted to accommodate a wide range of sample geometries, including typical cryo vials and thin-film samples. Thermal behavior of the device was investigated through computer-based modeling, which guided iterative design improvements under various operating conditions. Thermal equivalent circuit models were employed to identify key parameters—such as material properties and thermal resistances—based on temperature data from partial prototype setups. These models were then validated using experimental measurements from fully assembled prototypes. For a more detailed understanding of spatial temperature variations within the components, finite element simulations were conducted. Initial tests have been performed under passive cooling conditions using saline model fluid. The next development stages will focus on implementing active heat flow, along with optimized temperature control strategies. These steps will form the basis for a cost-effective and flexible platform for cryopreservation

SWEET, SALTY AND CROWDED: CHALLENGES IN CRYOPRESERVING AQUATIC SPERM. EXPLORING OSMOTIC STRESS AND THE "SALT INTOXICATION" THEORY IN PRACTICE - FROM STURGEONS TO CORALS

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ABSTRACT

Cryopreservation of aquatic sperm requires balancing cellular integrity with osmotic stress mitigation. According to the salt intoxication theory, excessive salt in the cryomedium disrupts ionic homeostasis, impairing motility and viability – especially in species adapted to lower salinity. Sugar supplementation counters this effect, with required concentrations depending on species-specific seminal plasma osmolality. In sturgeons (<100 mOsm/kg), ~100 mM sugar balances osmotic pressure; salmonids (~300 mOsm/kg) require ~200 mM; corals, whose sperm are released into seawater (~1000 mOsm/kg), need ~0.9 M sugar to offset salt-induced stress. Interestingly, optimal sperm concentration also correlates with seminal plasma osmolality. Literature data (1,2,3) show salmonids – having intermediate osmolality – tolerate the highest densities, with peak post-thaw motility at $1–2 \times 10^9$ sperm/mL. Sturgeons and corals, in contrast, perform best at $0.5–2 \times 10^8$ sperm/mL, suggesting sperm from hypo- and hyper-osmotic environments are less suited to high-density storage. The underlying mechanism is unclear, but sperm morphology may play a role. Larger sperm, such as in sturgeons, have greater surface area-to-volume ratios and longer flagella, increasing membrane exposure to osmotic and ionic stress, and making them more vulnerable in crowded suspensions where metabolite buildup, ionic shifts, and collisions occur. Smaller, more compact salmonid sperm can be packed more densely without excessive damage or competition, supporting higher optimal concentrations. Coral sperm, though small, are adapted for immediate dispersal in open seawater, not dense aggregation, which may explain their lower crowd tolerance in cryoprotectants. This mini-review highlights the interplay between osmotic balance, cryoprotectant composition, and sperm morphology. Tailoring cryopreservation protocols to species-specific osmolality and structural traits is essential for maximizing post-thaw sperm quality in aquatic species.

REFERENCES

1. Judycka S, Nynca J, Liszewska, E, Dobosz S, Grudniewska J & Ciereszko A (2018) *Aquaculture* **486**, 90–97. https://doi.org/10.1016/j.aquaculture.2017.12.019
2. Kodzik N, Ciereszko A, Judycka S, Słowińska M, Szczepkowska B, Świderska B & Dietrich MA (2024) *Sci Rep* **14**, 17707. https://doi.org/10.1038/s41598-024-17707-9
3. Ohki S, Morita M, Kitanobo S, Kowalska AA & Kowalski RK (2014) *Cryobiology* **69**, 489–495. https://doi.org/10.1016/j.cryobiol.2014.06.005

MUSSEL CRYOPRESERVATION, ADVANCES TOWARD APPLICATION AT MINI-HATCHERY SCALE

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ABSTRACT

Mytilus galloprovincialis is the most cultured aquatic species in Spain, 95% of its production is concentrated in Galicia. However, in recent years, its production has decreased due to climate change and the presence of invasive species. The seed of this bivalve, traditionally, is collected from the natural environment but now this source is insufficient. To solve this problem, this study proposes the production of mussel seed through aquaculture and the application of cryopreservation to ensure the availability of seed in mussel aquaculture. Currently, there is already an effective cryopreservation protocol published by Heres (1) but it only allows for the cryopreservation of very small volumes (0.25 ml straws) that cannot sustain a hatchery scaling. Expanding cryopreservation capacity remains a challenge, and a logical first step involves comparing the performance of different freezers with larger capacity. The goal is to assess differences in cryopreservation efficiency and determine whether adjustments to the current protocol are necessary to optimize it for the larger capacity freezers. The experimental protocol follows the methodology outlined by Heres et al.(1). D-larvae at 72 hours post-fertilization were selected, concentrated, and mixed at a 1:1 ratio with a cryoprotectant solution containing 10% ethylene glycol and 0.4 M trehalose. After a one-hour equilibration period, the larvae were placed in straws and loaded into the respective freezers, where they were cooled at a controlled rate of -1°C per minute. Upon reaching -35°C, the straws were plunged into liquid nitrogen and stored until thawing. Thawing was performed by immersing the straws in a 35°C water bath for six seconds. In total, 5 male/female crosses (72-hour D-larvae) were cryopreserved and the results showed that the outcome of the cryopreservation presents differences in larval normality. Therefore it is necessary to make changes in the current protocol to scale production using these new freezer.

REFERENCE

1. Heres P, Troncoso J & Paredes E (2022) *Sci Rep* **12**, 13632 https://doi.org/10.1038/s41598-022-17935-0

FIRST SUCCESSFUL CRYOPRESERVATION OF A JELLYFISH LARVA

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ABSTRACT

Conservation of marine species through cryopreservation plays a crucial role in protecting biodiversity, managing populations sustainably and enhancing aquaculture practices. Furthermore, its use in marine model organisms offers valuable opportunities for advancing biological research. While established methods exist for freezing sperm, embryos, and larvae of several marine invertebrates, the cryopreservation of cnidarians, particularly jellyfish, remains largely understudied. In this study, we report for the first time the successful cryopreservation of a cnidarian larva, focusing on the ephyrae, the initial larval stage of *Aurelia aurita* (Linnaeus, 1758), a jellyfish characterised by an exceptionally high water content (96.3%). This trait presents specific challenges in understanding the interactions between seawater properties and cryoprotective agents in organisms with high hydration levels. Drawing from the limited data available on cnidarian sperm (1) and cell culture experiments (2), we developed a series of toxicity assays with DMSO at the concentrations ranging from 0.5 to 6 M to establish optimal equilibration period. Successful cryopreservation was achieved using 1.5 M DMSO combined with 0.04 M trehalose, and 1% BSA as a post-thaw treatment, yielding 100% survival immediately after thawing and 33% survival after 48 hours. Live-dead fluorescence staining was employed to assess cellular damage caused by the process. Currently, we are working on evaluating long-term survival to determine the extended viability of these

protocols. This study opens new avenues for the conservation and study of high-water content marine species.

REFERENCES

1. Huene AL, Koch JC, Arregui L, Liu Y, Nicotra ML, Weis VM & Tiersch TR (2022) *Animals* **12**, 2537. https://doi.org/10.3390/ani12192537
2. Fricano C, Röttinger E, Furla P & Barnay-Verdier S (2020) *Cells* **9**, 2541. https://doi.org/10.3390/cells9122541

CRYOPRESERVATION AS A STRATEGIC TOOL FOR THE MUSSEL AQUACULTURE INDUSTRY

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ABSTRACT

Mussel production is one of the biggest aquaculture industries in Spain and the region of Galicia produces 97% of total aquaculture production in this mollusk. In this region mussel aquaculture has a high socio-economic impact. Most of the juvenile harvesting is recollected from natural sources in a traditional way, but lately its harvesting has opened conflicts with other species, biodiversity conservation and ecosystem services, mostly with the goose barnacle harvesting sector. In addition, the seasonal variability in the upwelling system of the area has introduced a factor of instability and uncertainty to the industry production that has been more acute in the last decade. Cryopreservation could be a tool to assist the mussel industry needs, as it allows continuous seed production for indoor aquaculture. In our lab, we have developed and standardised a reliable mussel larvae cryopreservation protocol (1) that we aim to transfer to industry through an R&D contract. The aim of this work is to present different strategies we plan to apply to the current mussel cryopreservation protocol to make it suitable for industry use. These include assessing quality and scalability, providing training to aquaculture technicians for successful implementation in mini-hatcheries, and building producer confidence in safety of inland mussel seed production. As an initial step, we have produced a large-scale mollusc cryopreservation review where the aim was to obtain information on the market trends, how cryobiology of molluscs has evolved and species and countries where this biotechnology is being applied in the aquaculture sector. This work will introduce the preliminary results of such review in the context of the mussel industry.

REFERENCE

1. Heres P, Troncoso J & Paredes E. (2022) *Sci Rep* **12**, 13632 https://doi.org/10.1038/s41598-022-17935-0

CRYOPRESERVATION OF FISH EMBRYOS: INSIGHTS FROM CYPRINODON VARIEGATUS

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ABSTRACT

To date, successful cryopreservation of marine fish embryos has only been achieved for Zebrafish (1). This is mainly due to the high internal water content and low membrane permeability of fish embryos, making them highly susceptible to intracellular ice formation when exposed to low temperatures. In this study, the species *Cyprinodon variegatus* was selected as an experimental model due to its remarkable resistance mechanisms, including diapause and broad thermal tolerance. Embryos were subjected to dehydration treatments using 2 M sucrose for varying exposure times (30 to 180 minutes) followed by addition of 1 M dimethyl sulfoxide (DMSO) during dehydration to evaluate its effect on survival and morphological development. Survival and external morphology assessments were conducted after 10 minutes, 3 days, and 5 days of incubation at 25°C. The results presented here confirmed the species' notable tolerance to osmotic stress induced by dehydration, as well as resistance to 1 M DMSO exposure. However, resistance significantly decreased with the increase of DMSO concentration to 2 M. Our first vitrification attempts were unsuccessful, with all embryos showing evidence of intracellular ice crystals in the yolk sac, likely causing immediate morphological abnormalities and the onset of cell death. Therefore, further optimisation of vitrification conditions is needed. In conclusion, *C. variegatus* demonstrates high potential as a model for embryo cryopreservation studies and proves broader resistance than zebrafish (Connolly et al., 2017).

REFERENCE

1. Connolly MH, Paredes E & Mazur P (2017) *Cryobiology* **78**, 106–109 https://doi.org/10.1016/j.cryobiol.2017.08.004

CRYOPRESERVATION OF COMPLEX MICROBIAL COMMUNITIES FROM ENVIRONMENTAL SAMPLES

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ABSTRACT

Cryopreservation of microorganisms is crucial in industrial and food applications to ensure microbial activity and key traits for consistent products or processes. This typically involves single species or low-diversity cultures that can be easily revived. Cryopreservation is vital for scientific research, where costly environmental samples are collected in remote locations. Long-term preservation of complex samples presents a challenge in maintaining structural diversity during revival. Here we look at cryopreserving and reviving complex microbial communities from both a hypersaline microbial mat (sediment) and coastal waters. Amplicon sequencing of the 16S and 18S rDNA was used to determine the community composition of bacteria and eukaryotes, respectively. Tests included the use of different cryoprotective agents (CPAs) and different durations of cryopreservation at −150 °C. For the sediment sample, it appears the CPAs cannot be separated from the preserved sediment samples without disturbing community structure, while carry over of these compounds may influence reconstitution of the communities. Although both glycerol and DMSO are good CPAs for microbial mat assemblages, carry over of these compounds had a profound negative effect on the reestablishment of a functional microbial mat. For seawater samples, as part of the Microbe project led by the RCC (https://roscoff-culture-collection.org), we developed a novel protocol to concentrate cells and to assess microbial community composition using millimetric volumes, as opposed to the liters required in more conventional approaches. Challenges for successfully cryopreserving complex microbial communities from different environments are discussed.

THE PROGRESS IN ESTABLISHING A CRYOBANK FOR CROP PLANT SPECIES IN POLAND

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ABSTRACT

Despite numerous studies aimed at the development of cryopreservation protocols for various plant species, liquid nitrogen is rather poorly implemented for the storage of crop plant germplasm in Poland. Regarding clonally propagated species, only collections of apple tree and garlic cultivars are safely stored under cryogenic conditions. To secure other collections, especially that of tetraploid potato, the Plant Breeding and Acclimatization Institute – National Research Institute (PBAI-NRI) in Radzików is currently setting up a central cryobank for Polish crops. Our cryopreservation and cryobanking activity is currently focused on three areas: 1. Physical arrangement of the in vitro laboratory and the cryobank. In accordance with the National Resilience and Facility Plan, the Recovery and Resilience Facility has provided the necessary funds for the acquisition of the complete cryogenic and in vitro laboratory equipment. By 2025, the public procurement is anticipated to be finalized. 2. The initiation of a cryogenic collection of tetraploid potato cultivars. Using the droplet vitrification technique adopted from the Leibniz Institute of Plant Genetics and Crop Plant Research in Gatersleben, Germany, the first potato cultivars were cryopreserved in 2024. Subsequent cultivars are continuously being acquired from the Potato Gene Bank In Vitro in the Bonin Division of the PBAI-NRI and propagated to be introduced into LN. 3. Research on the influence of cryopreservation on the genetic and epigenetic stability of plants. The AFLP and MSAP markers, together with HPLC-RP, were used to investigate whether cryopreservation induces variability at the level of DNA sequence and methylation in gentian somatic hybrid plants and whether the level of variability depends on the cryopreservation method. A comparison was made between plants that were cryopreserved through encapsulation-vitrification and encapsulation-dehydration and those that were not. The alterations in cytosine methylation levels were observed in plants that had undergone cryopreservation using both methods.

UTILIZING ALGINATE HYDROGELS TO ENHANCE CRYOPRESERVATION EFFICIENCY IN PEACH (*PRUNUS PERSICA*) SHOOT TIPS

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ABSTRACT

The long-term conservation of *Prunus persica* (peach), a fruit crop of major commercial and genetic importance, is hindered by the species' limited response to conventional cryopreservation methods (1). Recalcitrance to dehydration and cryoprotectant toxicity often leads to low survival and regrowth rates, posing significant challenges for the preservation of clonal germplasm. This study explores a novel cryopreservation approach for peach shoot tips using a modified D-cryoplate technique (2, 3), enhanced by the inclusion of Pluronic F-68, a non-ionic surfactant known for its membrane-stabilizing properties. *In vitro*-grown shoot tips (2–3 mm) from six peach genotypes were precultured in stepwise sucrose concentrations and then embedded in a thin layer of sodium alginate hydrogel directly on aluminium strips. This hydrogel matrix acted not only as a mechanical stabilizer during cryoprotectant exposure and liquid

nitrogen immersion but also as a modulator of water loss, enabling more controlled and uniform dehydration (3). Cryoprotection was performed using a loading solution and PVS3, both with and without Pluronic F-68. Following rapid cooling in liquid nitrogen and subsequent thawing, shoot tips were unloaded and transferred to recovery medium for regeneration assessment. The integration of alginate hydrogels significantly improved shoot tip regrowth, particularly when combined with Pluronic F-68. The highest regeneration rates–up to twice that of untreated controls–were observed in samples receiving both treatments. The D-cryoplate platform, with its fixed physical support and defined dehydration environment, further contributed to improved survival by minimizing tissue handling and optimizing vitrification dynamics. These findings confirm the synergistic benefits of using alginate hydrogels and surfactants within structured cryopreservation systems. The modified D-cryoplate method offers a reproducible and scalable solution for the long-term storage of peach and other cryo-sensitive plant species.

REFERENCES

1. Damiano C, Sgueglia A, Arias M, Frattarelli A, Condello E & Caboni E (2011) *Acta Hortic.* **918**, 121-124 https://doi.org/10.17660/ActaHortic.2011.918.13
2. El Merzougui S, Benelli C, El Boullani R & Serghini MA. (2023) *Plants* **12**, 2143 https://doi.org/10.3390/plants12112143
3. Arizaga MV, Navarro OFV, Martínez CRC, Gutiérrez EJC, Delgado HAL, Yamamoto S, Watanabe K &Takao N (2017) *The Horticulture Journal* **86**, 222-228 https://doi.org/10.2503/HORTJ.OKD-002

FROST RESISTANCE DIFFERS IN ANTARCTIC AUTOTROPHS ACCORDING TO THEIR HYDRATION STATUS: COMPARATIVE STUDY OF REPRESENTATIVES OF CHLORO- AND CYANOLICHENS

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ABSTRACT

Their ability to resist to sub-zero temperature, however, depends on actual content of water in their bodies. The resistance increases with dehydration and is considered high when the organisms are exposed to sub-zero temperature in dry state. To address species-specifity of the cryoresistance, we performed a series of lab experiments in two Antarctic lichens (1) *Xanthoria elegans*, collected in James Ross Island, Antarctica, and (2) *Lecania brialmontii*, King George Island, Antarctica. Frost resistance of fully-hydrated samples was evaluated by several chlorophyll fluorescence (ChlF) parameters evaluating primary photochemical processes of photosynthesis. We used ChlF imaging method (Fluorcam HFC-010, Photon Systems Instruments, CZ) and evaluated the following parameters: (1) maximum yield of photosystem II (Fv/Fm), (2) effective quantum yield (FPSII), and (3) photochemical quenching (qP). Samples were measured at 24°C first, then air dried to fully dry state and shock-frozen in liquid N. After removal, the samples were warmed up to 24 °C (120 min) and then rehydrated for 24 h. Then, ChlF parameters were measured again and the frost-induced limitation evaluated as a difference between the values recorded ´before´ and ´after´ immersion into liquid N. Our data suggest that all investigated species are highly cryoresistant if they are exposed to –198ºC in dry state. In such case, Fv/Fm, FPSII, and qP decreased to only very limited extent. The liquid N-induced decrease in the parameters was, however much more pronounced, if the organisms were partly hydrated (range of relative water contents 10-30 %). The shock freezing-induced decrease in

ChlF parameters were attributed to ice crystals formation and direct damage to cellular structures, pigment-protein complexes and thylakoid membrane components in chloroplasts in particular.

FEATHERS ON ICE: ADVANCES IN AVIAN SEMEN CRYOPRESERVATION FOR BIODIVERSITY CONSERVATION

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ABSTRACT

Semen cryopreservation is a key element of avian reproductive biotechnology, increasingly recognized as an essential strategy in biodiversity conservation programs. Currently, over 4,000 species of mammals and birds are classified as vulnerable, endangered, or critically endangered on the IUCN Red List — a figure that continues to rise. This alarming trend underscores the need for innovative tools to safeguard genetic diversity and prevent extinction. Assisted Reproductive Techniques (ART), including semen cryopreservation and artificial insemination, have emerged as powerful solutions to address challenges such as habitat loss, poaching, and genetic bottlenecks. In birds, ARTs face unique challenges due to the structural and physiological characteristics of avian gametes. Avian spermatozoa exhibit high membrane fluidity, elongated morphology, and low cholesterol-to-phospholipid ratios, making them particularly susceptible to cryoinjury. Furthermore, cryopreservation of avian oocytes and embryos remains largely impractical due to their large size and lipid-rich content, positioning semen preservation as the primary focus for avian genetic banking. Recent advances in cryoprotectant strategies have significantly improved post-thaw sperm quality. Alternative permeating cryoprotectants such as dimethylacetamide (DMA), dimethylformamide (DMF), and N-methylacetamide (MA) offer superior results compared to glycerol, which exhibits contraceptive effects in birds. Non-permeating cryoprotectants like trehalose further stabilize sperm membranes and reduce osmotic stress during freezing and thawing. Additionally, novel approaches such as nanotechnology-based delivery systems are being explored to enhance protection, though their application in avian species remains limited. Our current research focuses on the African penguin (*Spheniscus demersus*), a critically endangered species. We have developed and optimized a protocol using DMF as a permeating cryoprotectant in combination with trehalose. This strategy aims to improve post-thaw sperm viability and motility, supporting the creation of a genetic resource biobank to facilitate future conservation breeding efforts. In conclusion, semen cryopreservation represents a promising and practical tool to preserve avian genetic diversity. Our work with the African penguin exemplifies how tailored cryoprotectant solutions can directly support global efforts to safeguard vulnerable bird populations for future generations.

INSIGHTS INTO CRYOPRESERVATION-INDUCED MOLECULAR ALTERATIONS IN TURKEY (*MELEAGRIS GALLOPAVO*) SPERMATOZOA

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ABSTRACT

Cryopreservation of turkey (*Meleagris gallopavo*) semen plays a vital role in preserving genetic diversity, supporting breeding programs, and advancing avian reproductive biotechnologies. However, turkey

spermatozoa are particularly sensitive to cryodamage, which results in reduced post-thaw motility, viability, and fertilizing capacity. Consequently, recent research has intensified efforts to optimize freezing protocols and to better understand the cellular and molecular mechanisms underlying cryodamage in turkey spermatozoa. Advancements in cryoprotective strategies include the use of both permeant (DMSO, DMA) and non-permeant agents (sucrose, trehalose, and Ficoll), often in combination with antioxidants such as glutathione or lycopene (1-4). The choice of extender, freezing rate, thawing temperature, and dilution ratio has also been shown to significantly influence post-thaw spermatozoa quality (1). Modern assessment methods have significantly improved our understanding of turkey spermatozoa cryodamage. So far, strategies such as cellular and functional assessments using flow cytometry, proteomic analyses via two-dimensional difference in-gel electrophoresis coupled with matrix-assisted laser desorption/ionization mass spectrometry and metabolomic profiling using nuclear magnetic resonance spectroscopy have been applied (5-7). At the Gamete Biology Group, we have investigated the molecular mechanisms responsible for the decline in the quality of cryopreserved turkey semen for over a decade. At the molecular level, we confirmed disruptions in proteins involved in energy metabolism, particularly those related to the tricarboxylic acid (TCA) cycle and oxidative phosphorylation. We demonstrated that the freezing–thawing process affects the assembly of spermatozoa flagella, primarily by disturbing the axoneme and outer dense fibers, and leads to alterations in proteins responsible for sperm–egg binding as well as those involved in the acrosome reaction (6). Knowledge of the molecular and cellular mechanisms of cryodamage in turkey semen is a prerequisite for improving semen preservation procedures. A deeper understanding of these cryodamage mechanisms allows researchers to develop targeted strategies to reduce cryoinjury and improve post-thaw sperm quality.

REFERENCES

1. Iaffaldano N, Di Iorio M, Miranda M et al. (2016) *Br Poult Sci* 57(2), 264-270
2. Di Iorio M, Rusco G, Iampietro R et al. (2020) *Animals* (Basel) **10**, 421 https://doi.org/10.3390/ani10030421
3. Izanloo H, Soleimanzadeh A, Bucak MN et al. (2022) *Theriogenology* **179**, 32-38 https://doi.org/10.1016/j.theriogenology.2021.11.010
4. Rosato MP, Centoducati G, Santacroce MP et al. (2012) *Br Poult Sci* **53**, 545-552
5. Slowinska M, Liszewska E, Judycka S et al. (2018) *Poult Sci* **97**, 3709-3717 https://doi.org/10.3382/ps/pey209
6. Pardyak L, Liszewska E, Judycka S et al. (2025) *Theriogenology* **243**, 117473 https://doi.org/10.1016/j.theriogenology.2025.117473
7. Paventi G, Di Iorio M, Rusco G et al. (2022) *Biology* (Basel) **11**, 642 https://doi.org/10.3390/biology11050642

IMPROVED CRYOPRESERVATION OF CHICKEN GONADAL GERM CELLS VIA VITRIFICATION

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ABSTRACT

Although oocyte and embryo cryopreservation in chickens remains unfeasible, preserving embryonic gonads provides a promising alternative. Gonadal germ cells (GGCs) within embryonic gonads can develop into functional sperm or eggs. When injected into sterile 2.5-day-old embryos, cryopreserved

GGCs can colonize recipient gonads and produce donor-derived gametes. However, freeze/thaw damage prolongs the in vitro culture of cryopreserved GGCs compared to fresh cells, emphasizing the need to improve gonad cryopreservation. This study presents a vitrification protocol that preserves functional GGCs and improves derivation rates (DR) compared to slow-freezing with STEM-CELLBANKER.G onads from 9- to 10-day-old GFP+ embryos were used. For slow freezing, 9 gonads were frozen and thawed following Hu et al. (1, 2). For vitrification, 24 gonads were loaded into acupuncture needles, sequentially exposed to two chilled cryoprotectant solutions (Solution 1: 7.5% DMSO, 7.5% ethylene glycol; Solution 2: 15% DMSO, 15% ethylene glycol, 0.5 M sucrose), 5 minutes each, then immersed in liquid nitrogen. Thawing involved three steps at 37 °C in decreasing sucrose concentrations (1 M, 0.5 M, 0.25 M). Gonads were cultured individually for three weeks; cultures with ~50,000 GGCs or more were considered successful. DR was calculated as (successful cultures / total cultures) × 100. One culture was used to inject fifteen 2.5-day-old non-GFP embryos with ~5000 GGCs each, assessing gonadal colonization after 7 days. Vitrification yielded higher ($p<0.01$) DR (58.3%) and average GGCs/gonad (9.9×10^4) compared to SCB (0% and 7.6×10^3). DRs were higher ($p<0.05$) for vitrified testes (66.7% vs. 0%) and left ovaries (62.5% vs. 0%), with no difference for right ovaries. Left ovaries showed an increase ($p<0.05$) in GGC yield (1.4×10^5 vs. 5.7×10^3). Among embryos surviving GGC injection (10/15), 80% showed successful gonadal colonization. In conclusion, we propose a vitrification protocol that improves GGC derivation and offers an alternative to slow-freezing with STEM-CELLBANKER.

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REFERENCES

1. Tuanjun Hu et al (2022) *eLife* **11**, e74036 https://doi.org/10.7554/eLife.74036
2. Tuanjun Hu et al (2024) *Poult Sci* **103**, 1-9 https://doi/org/10.1016/j.psj.2024.104260

EFFECTS OF L-CARNITINE AND SERICIN ON SEMEN CRYOPRESERVATION IN ROOSTERS

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ABSTRACT

It has long been known that avian sperm are much more susceptible to cryopreservation than mammalian sperm, and it is also well known that spermatozoa are subjected to high levels of stress during freezing. To counteract this stress effect, the antioxidant defence system can be supported by the use of various antioxidants. In the present study, we investigated the effect of L-carnitine and sericin as antioxidants in extenders on semen cryopreservation. The pooled fresh semen was divided into four parts. The control (K) sample was diluted with Lake-diluent, the L-carnitine supplemented (C) group with 2mM/100ml L-carnitine, the sericin supplemented (S) group with 0.25 w/v% sericin mixed in the Lake-diluent, and the combined effects of antioxidants in the sericin-L-carnitine supplemented (C+S) group were investigated. After precooling at 5 °C for 30 min, 6% DMA was used as cryoprotectant, and the samples were deep-frozen for 15 min at 5 cm and for another 15 min at 1 cm above the surface of liquid nitrogen. Thawing was carried out in a water bath at 3.5 ºC for 1 min. It was determined in fresh and frozen/thawed samples motility by CASA system, viability and morphological integrity by aniline-eosin vital staining, DNA fragmentation by TUNEL Assay and acrosome reactivity by in vitro membrane assay. L-carnitine (C) and sericin (S) supplementation neither improve sperm motility nor increase the proportion of live, intact cells,

but reduced the incidence of DNA fragmented cells and preserved sperm acrosome reaction compared to the control (K) group. The combination of L-carnitine and sericin (C+S) was able to protect against DNA damage compared to the control (K) group, but no significant difference was found in other parameters. To investigate the impact of the positive results obtained on fertility, it is essential to carry out AI experiments.

BUILDING AN EFFECTIVE SYSTEM OF EX SITU CONSERVATION IN POLAND: CHALLENGES AND PROGRESS

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ABSTRACT

Climate change, agricultural intensification, and global threats to local animal genetic resources – including epidemiological risks – have increased the importance of ex situ conservation. Poland has a long tradition in this field. In 1968, the National Research Institute of Animal Production (IZ–PIB) established the first semen bank in Poland and one of the first in Europe. Ex situ efforts complement in situ actions, which in Poland cover 89 breeds, lines, and strains. In recent years, significant progress has been made: over 60,000 genetic samples have been collected, primarily from conserved breeds and partially from commercial ones. Modern cryopreservation technologies introduced at IZ–PIB enabled the conservation of poultry, fur animals, and bees. A funding mechanism was established to secure reproductive material from conservation bulls, involving cattle breeding stakeholders. Efforts are now underway to develop similar mechanisms for pigs and horses. Ex situ conservation in Poland focuses on storing semen, embryos, somatic cells, and bee genetic resources in specialized facilities such as the National Gene Bank. Collections are documented in national databases, with selected information shared internationally (e.g., EFABIS, EUGENA). Despite this infrastructure, key challenges remain: insufficient samples for some breeds, unstable funding, limited access to donor data, and inconsistent legal frameworks. Effective conservation requires a coordinated strategy involving scientists from various fields, breeders, policymakers, and public administration. Work is underway on a new national strategy to align actions and improve regulatory coherence. International collaboration and harmonized standards for data sharing and biological material management are increasingly important. National Bank of Biological Material is part of the European gene bank network EUGENA. Safeguarding animal genetic resources ex situ is not only a technical task, but also a strategic mission of national and social importance. Ongoing efforts are laying the foundation for a future integrated system that addresses both domestic and global conservation needs.

ADVANCES IN DEVELOPMENT OF THE EUROPEAN GENEBANK NETWORK FOR ANIMAL GENETIC RESOURCES

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ABSTRACT

The European Genebank Network for Animal Genetic Resources (EUGENA) brings together 27 genebanks from 11 European countries dedicated to the ex situ conservation and sustainable use of genetic resources from domesticated animal species (1). This European bio-repository for breeding and scientific research includes collections comprising 5,336,856 samples of semen, embryos, stem cells, primordial germ cells, DNA, blood, tissues, hair, and others. EUGENA is a crucial infrastructure for the long term conservation of animal genetic diversity, therefore each member genebank has to be officially recognized by the competent national authority as a facility for ex situ conservation. Established in 2016, the network is supervised and supported by the European Regional Focal Point for Animal Genetic Resources (ERFP), with main aim to foster collaboration between the member genebanks, improve the efficiency of developing the ex situ in vitro collections, and support harmonized acquisition and access terms for cryoconservation. As part of its objectives, EUGENA provides, via a web portal, one entry point to the member genebanks' sites and presents to the public the current state of their collections. This uniform set of data by breeds and material types is following the FAIR principles, enabling data sharing with other systems, thus supporting the countries in monitoring the SDG indicator 2.5.1b. To keep interoperability with the Domestic Animal Diversity Information System (DAD-IS) of the Food and Agriculture Organization of the United Nations, EUGENA was recently extended with new tools allowing for automated synchronization of the vocabularies of species, breed names, and transboundary breed names from DAD-IS. The new setup allows the network to handle situations where the collections contain material from breeds present in DAD-IS, sub populations of a breed, and from breeds which do not exist anymore. It also includes data export at the national level for systematic upload into DAD-IS.

REFERENCES

1. https://www.eugena-erfp.net

DEVELOPMENT OF ALTERNATIVE CRYOPROTECTANTS FOR ENHANCED CELL VIABILITY

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ABSTRACT

Cryopreservation is vital for assisted reproductive technology, stem cell therapies, and species conservation (1-5). It relies on cryoprotective agents (CPAs) to prevent freezing damage, yet conventional CPAs like dimethyl sulfoxide (DMSO) and glycerol (GLY) are toxic and ineffective for many cell types, limiting their clinical and research applications (1,2,6,7). This study explores how different cooling and thawing rates, combined with various cryoprotectants, affect cell viability. Traditional CPAs were compared with deep eutectic solvents (DES), assessing their thermal behavior, toxicity, and cell permeability. Preliminary findings suggest that certain DES formulations may enhance cryoprotection while reducing toxicity (8,9). Using advanced microscopy and precision-controlled thermal techniques, we analysed cellular responses to freezing and thawing in real time, providing insights into ice formation, structural changes, and post-thaw survival. Cooling and thawing rates were optimized to improve cryopreservation outcomes across different cell types. Understanding biophysical properties is crucial for developing optimised protocols. Cells with large osmotically inactive volumes may require slower CPA addition, while low-permeability cells may need extended incubation times. Measuring these parameters allows for more effective preservation strategies. This research advances cryopreservation by introducing alternative CPAs, particularly DES, to overcome the limitations of conventional methods. Optimizing cryoprotectant formulations and freezing protocols can improve post-thaw cell function, benefiting biomedical and biotechnological applications.

REFERENCES

1. Jang TH et al (2017) *Integr Med Res* **6** 12-18 https://doi.org/10.1016/j.imr.2016.12.001
2. Whaley D (2021) *Cell Transplant* **30**, 963689721999617 https://doi.org/10.1177/0963689721999617
3. Bolton RL et al (2022) *Reprod Fertil* **3** , 121-146. https://doi/org/10.1530/RAF-22-0005
4. Kaviani B & Kulus D. (2022) *Biology* **11,** 847 https://doi.org/10.3390/biology11060847
5. Engdawork AT. Belayhun & Aseged T (2024) *Ecol Genet Genomics* **31**, 100250 https://doi.org/10.1016/j.egg.2024.100250
6. Murray KA & Gibson MI (2022) *Nat Rev Chem* **6**, 579-593 https://doi.org/10.1038/s41570-022-00407-4
7. Bryant S et al (2021) *J. Colloid Interface Sci* **603**, 491-500. https://doi.org/10.1016/j.jcis.2021.06.096
8. Casciani V et al (2023) *Fert Ster* **120**, 506-520. https://doi.org/10.1016/j.fertnstert.2023.06.005
9. Bryant SJ et al (2022) *J Mater Chem B* **10**, 4546-4560 https://doi.org/10.1039/D2TB00573E

INNOVATIVE CRYOPRESERVATION AND REWARMING TECHNIQUES FOR CORAL LARVAE: ESTABLISHING A REGIONAL CRYOBANK NETWORK IN THE CORAL TRIANGLE

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ABSTRACT

Coral reefs worldwide are undergoing significant degradation due to various anthropogenic and environmental stressors, leading to a considerable decline in coral populations. Cryopreservation of coral genetic material has emerged as a critical strategy for conserving coral diversity. This approach enables the long-term preservation of the genetic pool of extant coral populations. In this study, we developed specialized cryopreservation devices designed to establish a coral larvae cryorepository through vitrification and nano-laser warming techniques. Thousands of larvae from reef-building corals have been cryopreserved in our facility, providing a viable genetic repository. The larvae can be rapidly re-warmed using a cryojig, which incorporates a high-precision nano-laser warming technique that achieves a laser accuracy of 95% and a rewarming success rate of 62%. To expand these efforts, we have established the first regional network of coral larvae cryorepositories within the Coral Triangle— a biodiversity hotspot hosting the highest coral species density globally, encompassing Taiwan, Thailand, Malaysia, Indonesia, and the Philippines. We project that these advanced techniques will enable the establishment of cryobanks across the participating nations. Post-thaw larvae can be re-cultured into mature corals, thus facilitating the restoration and enhancement of reef biodiversity. We anticipate that our innovative freezing technologies will play an essential role in the cryopreservation of valuable coral species, contributing to the long-term sustainability of coral reefs worldwide.

MARINE CRYOPRESERVATION: FROM MICROBES TO INVERTEBRATES

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ABSTRACT

Since the discovery in the late 1940s of the ability to cryopreserve fowl sperm, cryopreservation has strongly impacted several domains of biological research. Important technical advances have been made, notably related to medical fields, to improve the cryopreservation of diverse biological samples, from human stem cells to animal gametes, and from plants to microorganisms and viruses. The Roscoff Culture Collection (RCC, https://roscoff-culture-collection.org/), one of the largest public culture collections of marine organisms in the world, has been developing a cryopreservation platform and associated services over the last 15 years. Of the ca. 10,000 RCC culture strains, over 4,000 have been successfully cryopreserved so far, mostly belonging to green algal lineages (e.g., *Chlorodendrophyceae, Trebouxiophyceae, Mamiellophyceae*), haptophytes (e.g., *Prymnesiophyceae*), stramenopiles (e.g., *Bacillariophyceae*) and cyanobacteria. In recent years, the technique has also been successfully applied to preserve algal viruses (myoviruses infecting *Synechococcus*, prasinoviruses infecting *Micromonas*), seaweeds (gametophytes and sporophytes of *Saccharina latissimi, Macrocystis pyrifiera* infected with the parasite *Eurychasma dicksonii*) and parasites of bivalves and dinoflagellates (*Perkinsus olseni, Perkinsus chesapeaki, Perkinsus chioggia, Dinomyces sp*. and *Parvilucifera* sp.). As part of the MICROBE project (https://www.microbeproject.eu/), the RCC is developing a novel protocol to preserve complex microbial communities from millimetric volumes of seawater samples. The success of preserving a full biological community would have significant advantages, such as preventing the loss of biodiversity due to climate change. We are currently also working on establishing a new research line on the cryopreservation of marine invertebrate gametes and embryos.

DESIGN AND VALIDATION OF A FLUIDIC CHAMBER FOR CPA/VA DIFFUSION IN A SINGLE OOCYTE USING RAMAN SPECTROSCOPY

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ABSTRACT

Effective cryopreservation of reproductive cells requires precise control of cryoprotectant and vitrification agent (CPA/VA) exposure, governed by their diffusion dynamics. Understanding CPA/VA kinetics at the single-cell level is essential for developing optimised protocols but remains technically challenging.

As part of the EU-funded CryoStore project, which aims to improve long-term preservation of reproductive cells through non-invasive, controlled cryopreservation strategies, we present the design and validation of a temperature-controlled fluidic chamber for real-time CPA/VA diffusion analysis in individual porcine and bovine oocytes using Raman spectroscopy. The chamber is engineered to integrate seamlessly with a high-resolution Raman microscope, ensuring stable sample positioning, sterility, and optical accessibility. Its sealed, leak-proof design prevents external contamination and is compatible with dynamic perfusion and temperature regulation from +22°C to −55°C, enabling simulation of physiologically relevant cryopreservation conditions. All components were modelled in SOLIDWORKS and fabricated at the institutional mechanical workshop. Validation included thermal stability tests, optical clarity assessment, and reproducibility of Raman signal acquisition during CPA perfusion. Reference solutions (e.g., DMSO, ethylene glycol) were used to confirm the system's suitability for tracking CPA diffusion kinetics over time. This validated setup represents a novel platform within the *CryoStore* framework for investigating intracellular CPA transport under defined conditions, supporting the development of more precise and less invasive cryopreservation protocols for reproductive cells. In the next phases of the project, this system will be applied to assess intracellular CPA diffusion in live porcine and bovine oocytes during controlled

cooling cycles. These experiments will be complemented by structural analysis of cryo-induced cellular alterations, including lipid content, membrane integrity, and aquaporin expression.

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REFERENCES

1. Hubenia O, Tewes R, Leal-Marin S, Winkler C, Iddi A, & Glasmacher D (2025) in *Abstract book. 49th Annual Conference of Young Scientists. Cold in Biology and Medicine*.

PUSHING THE LIMITS OF SPERM IMAGING: DIRECTIONAL FREEZING MEETS HIGH-RESOLUTION MULTIPHOTON TECHNOLOGY

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ABSTRACT

The CryoStore EU-project "Innovation in Germplasm Cryopreservation for Improved Animal Breeding and the Conservation of Europe" intends for the optimization of cell freezing with new protocols and high-resolution imaging. High-resolution imaging of frozen sperm cells is a major bottleneck in reproductive biotechnology and genetic conservation (1-3). The presented study focuses on "the development of a cryostage for two-photon microscopy enabling real-time imaging under controlled thermal gradients during cryopreservation with directional freezing. The cryostage was designed using Solidworks2024TM. Viability of ram sperm cells received from an EU-approved Semen Collection Center (D-KBS2-003 EWG) was assessed using Calcein AM (2 µg/ml) and Propidium Iodide (10 µg/ml) and imaged using a 1068 nm laser two photon microscope. The cryostage incorporates a copper-based sample holder, embedded heating elements, resistive temperature detectors, and a liquid nitrogen cooling system controlled via a Linkam LNP pump. Optical access is maintained through a single window aligned with a high-numerical-aperture objective. PI-labeled non-viable cells were detected, Calcein AM signals were not seen under the two-photon microscope— poor spectral overlap with the two-photon laser. Thus, we will use a 780 nm laser suitable for dyes with blue excitation spectra and dyes better matched to 1064 nm excitation like 532 nm linear excitation. Further work will focus on improving staining protocols, temperature control, validating the real-time imaging of ice dynamics and cell viability during freezing. The developed platform will then advance research in the field by making available a means for reproducible, high-resolution cryomicroscopy of reproductive cells.

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REFERENCES

1. Bedrossian M, Deming J. W, & Nadeau J (2018) *Methods Microbiol* **45**, 293–321 https://doi.org/10.1016/BS.MIM.2018.05.001
2. Gandini L, Pallotti, F, Paoli D & Lenzi A (2019) *Endocrinology* (Switzerland) 1235–1250 https://doi.org/10.1007/978-3-319-44441-3_41
3. Mutsenko V, Chasnitsky M, Sirotinskaya V, Müller M, Glasmacher B, Braslavsky I, & Gryshkov O (2021). *IFMBE Proceedings* **80**, 391–398. https://doi.org/10.1007/978-3-030-64610-3_45

A COMPARATIVE REVIEW OF DIFFERENT CASA SYSTEMS FOR SPERM QUALITY ASSESSMENT IN MARINE CRYOPRESERVATION

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ABSTRACT

The Computer-Assisted Sperm Analysis (CASA) system is widely used to capture and evaluate sperm quality, with particular emphasis on motility as a key indicator. This analysis is performed using images obtained via microscopy. OpenCASA, is an alternative to CASA that offers similar analytical capabilities through freely available open-source software. The primary aim of this review was to analyse, compare, and assess the advantages and limitations of both systems, specifically in the context of cryopreservation in marine organisms. The literature review revealed that both systems commonly focus on the assessment of sperm velocity across a diverse range of marine species, with fish representing the most frequently studied group. Notably, the reviewed studies often lacked consistent objectives, highlighting the adaptability of both systems for a variety of research purposes. OpenCASA, in particular, stands out for its flexibility and cost-effectiveness, as it does not require paid updates from commercial providers. Instead, updates are developed and shared by the OpenCASA user community in response to evolving research needs. The findings indicate that OpenCASA is especially suitable for research scenarios with limited measurement needs and high technical training, offering accurate results at a lower cost, albeit with some calibration requirements. In contrast, for commercial or high-frequency use, the proprietary CASA system – with its integrated accessories and technical support – may offer greater efficiency and speed in data acquisition.

STANDARDIZED HANDLING AND TEMPERATURE MONITORING OF CRYOPRESERVED SAMPLES IN CRYOBANKS

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ABSTRACT

Cryobanks are critical infrastructures for the long-term preservation of biological materials such as cells, tissues, reproductive material, and microorganisms. Ensuring sample viability during storage at ultra-low temperatures (typically < –150 °C) is essential for biomedical research, biodiversity conservation, and

healthcare applications. While freezing protocols are generally well-optimized, the handling of samples—particularly during loading, unloading, or transport—remains a vulnerable process where human error and inconsistent practices can lead to unintended temperature rises and potential damage. To address this, our project aims to systematically evaluate temperature changes during routine handling procedures across different biobanks and assess the impact of these fluctuations on sample integrity. This collaboration between the institute for multiphase processes (IMP) and the alliance of german cryobanks (GDK) is structured into three working steps: 1. Status Quo Assessment: A questionnaire was distributed among project partners to gather detailed information on container types, storage methods, handling routines, and terminology. This phase provided the basis for selecting representative model systems for further study. 2. Temperature Measurements: Model systems were recreated at IMP and IPK to simulate typical handling scenarios. Using embedded sensors and differential scanning calorimetry (DSC), we measured internal sample temperatures and determined critical parameters such as glass transition temperatures of various cryoprotectants. 3. Impact Assessment and Guidelines: To evaluate the consequences of temperature increases, selected model samples were subjected to defined exposures and returned to partners for quality assessment. Based on the findings, practical recommendations and standardized guidelines for safe handling and transport were developed. This project highlights the importance of harmonized working procedures in cryobiobanking and provides data-driven strategies to reduce risk and safeguard sample quality during every stage of handling.

QUERCETIN OR ITS CLATHRATE FORMS: WHAT TO CHOOSE FOR HYPOTHERMIC STORAGE OF FISH?

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ABSTRACT

Logistical problems in wartime and violation of storage conditions of perishable products prompt the search for effective methods of preserving their quality (1). One of the most promising areas is the use of natural flavonoids, in particular quercetin (Q), which has powerful antioxidant and antimicrobial properties. However, the low bioavailability of quercetin due to poor solubility in water limits its practical use. To increase the solubility and bioavailability of quercetin, we synthesized supramolecular clathrate complexes: one with 2-hydroxypropyl-beta-cyclodextrin (QC) and the other with dehydroquercetin (DQC).The effectiveness of the protective effect of natural quercetin Q, QC and DQC on the muscle tissue of Cyprinus carpio during hypothermic storage at a temperature of 4±1°C for 9 days was evaluated. For this purpose, carp muscle tissue (*Cyprinus carpio*) was kept in aqueous solutions of Q, QC and DQC with different concentrations, then stored under specified conditions. To assess the nutritional quality of the studied fish samples, we selected the following indicators: organoleptic indicators (smell, color, appearance), uric acid content as a marker of autolytic processes (biochemical method) and microbiological assessment (smear-imprint method). We showed that the treatment of carp muscle tissue with QC and DQC solutions is effective at concentrations of 0.4 and 0.2 g/l for QC and DQC, respectively, in contrast to Q solution at the same concentrations. The use of clathrate forms of quercetin allowed to increase the shelf life of fish muscle tissue at a temperature of 4±1°C for 9 days by more than two times.Therefore, our results suggest that among Q, QC and DQC, soluble quercetin clathrate complexes are effective in terms of shelf life of *Cyprinus carpio* muscle tissue. However, DQC requires a lower concentration of active ingredient for synthesis, and its efficacy is similar to QC in terms of autolytic processes (uric acid content).

REFERENCES

1. Zhiliang Huanga, Qi Wanga, Jun Caoa, Dayong Zhoub, Chuan Lia (2024) *Critical Reviews in Food Science and Nutrition* **64(18)**, 6298-6317.

EFFECT OF HYDROXY-SUBSTITUTED CHALCONES ON THE QUALITY OF CRYOPRESERVED CANINE SEMEN

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ABSTRACT

Chalcones are naturally occurring compounds with diverse biological activities, including antioxidant, anti-inflammatory, antimicrobial, and anticancer effects. Their chemopreventive properties involve modulation of metabolic enzymes and inhibition of pro-oxidative pathways, such as lipoxygenase. Due to the high susceptibility of sperm membranes to cryo-induced damage, particularly lipid peroxidation (LPO) -hydroxychalcones may offer protective effects during semen preservation. This study aimed to evaluate the effects of selected hydroxychalcones on the quality of canine spermatozoa after cryopreservation. A comprehensive assessment was conducted, including motility parameters (CASA), viability, acrosome status, mitochondrial membrane potential, lipid peroxidation, and chromatin integrity. In two experiments, canine semen was incubated with selected hydroxy-substituted chalcones: (Exp I) chlorinated chalcones—2'-hydroxy-2-chlorochalcone (2ClCH) and 2'-hydroxy-4-chlorochalcone (4ClCH); (Exp II) methylated analogs 2'-hydroxy-2-methylchalcone (2CH3CH) and 2'-hydroxy-4-methylchalcone (4CH3CH), at 0.1 and 0.4 mM, then subjected to cryopreservation. Sperm quality was assessed at 0, 2, and 4h post-thaw using CASA system and flow cytometry. In Exp I, no significant effects of treatment were observed on motility or other sperm parameters, including LPO. However, viability differed significantly between groups and over time. Although post-hoc tests showed no significant pairwise differences vs. control, 4ClCH 0.4 consistently showed the highest percentage of dead sperm at all time points, suggesting potential cytotoxicity. In Exp II, all tested groups significantly reduced the proportion of LPO-positive dead spermatozoa at 2 and 4h, and 4CH3CH reduced the proportion of LPO-positive live spermatozoa at 2h, indicating a protective effect. However, both methylated compounds also significantly impaired motility parameters (VAP, VSL, total and progressive motility) at 2h and 4h post-thaw. In conclusion, selected hydroxychalcones showed both beneficial and adverse effects on post-thaw sperm quality. While antioxidant protection was noted (especially for 4CH3CH), reduced motility raises concerns about their applicability in cryopreservation protocols. Further studies are needed to clarify mechanisms and refine dosing strategies.

FIRST EFFECTS OF FROZEN OVARIAN TISSUE RETRANSPLANTATION FOLLOWING FREEZING PROGRAM WITH DIMETHYLSULPHOXIDE (DMSO) AS A CRYOPROTECTIVE AGENT (CPA)

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ABSTRACT

Fertility preservation in female patients who have to be subjected to extensive medical treatments (regardless of the medical reasons) may be accomplished in the first place using oocyte or embryo cryopreservation, but eventually is achieved through ovarian tissue freezing or vitrification. At 'nOvum' Fertility Clinic, we initiated a program of ovarian tissue cryopreservation in 2011. Following our excellent data on embryo freezing with DMSO as the CPA (1), we adapted this freezing solution and a long-curve freezing program to tissue freezing as well. The staining of selected control samples by neutral red dye (2) showed a significant portion of surviving follicles in the tissue. By now, we have stored tissue samples from 35 patients; however, the first patient attempted the retransplantation trial only recently. Ovarian tissue collected laparoscopically from one ovary transported on ice/water to the laboratory, was subjected to processing on a cold plate according to the standard procedure. Ovarian cortex released from stroma tissue was divided into small pieces (2-3 x 5 mm) and saturated in cold freezing solution consisting of 1.5 molar DMSO dissolved in supplemented Leibovitz (L-15) medium. Within 30 minutes, 2-3 pieces of tissue were allocated to 1.5 mL cryovials, frozen slowly to -80°C, and then moved to liquid nitrogen for storage. In due time, thawing was executed by exposing a selected number of vials to air for approximately 12 minutes. Rehydration was performed in 3 concentrations of trehalose solution (0.5, 0.25, and 0.0 molar), 10 min each at RT. Nine strips were transplanted laparoscopically into the second, previously unoperated ovary. After 28 days of transplantation, menstrual symptoms were observed, followed by the growth of follicles detected ultrasonographically. Both phenomena indicate the resumption of the tissue's functional activity. It preliminarily confirms the efficiency of the procedure performed.

REFERENCES

1. Papis K, Stachowiak E, Lewandowski P & Kozioł K (2013) *Rep Biol* **13**, 28-29. https://doi.org/10.1016/j.repbio.2013.01.075
2. Kristensen S. G, Liu Q, Mamsen L. S, Greve T, Pors S. E, Bjørn AB, Ernst E, Macklon KT & Andersen CY (2018) *Hum Rep* **33**, 2276–2284. https://doi.org/10.1093/humrep/dey318

CRYOPRESERVATION OF FISH SPERM: 50 YEARS OF RESEARCH AND CRYOBANK DEVELOPMENT AT THE INSTITUTE FOR PROBLEMS OF CRYOBIOLOGY AND CRYOMEDICINE (UKRAINE)

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ABSTRACT

Cryopreservation of gametes and genomes in specialized cryobanks is recognized as a key tool for preserving genetic resources and enabling future species restoration (1). Since 1973, under the leadership of Dr. Yevgen Kopeika, the Institute for Problems of Cryobiology and Cryomedicine (IPC&C) has developed and refined cryopreservation methods for fish sperm. In 1976, the first successful protocol for sturgeon sperm was established, followed by carp sperm cryopreservation in 1979. Over five decades, IPC&C has expanded its research to over 40 fish species and breeds and developed a cryobank housing extensive genetic material from commercially important species. Cryobank also preserves from diverse fish species, including rare sturgeons such as the Aralsky ship *Acipenser nudiventris* and Great (white) sturgeon *Huso huso*. Recent studies have focused on the fundamental biophysical mechanisms underlying sperm cryopreservation, including investigations of osmotic stress and its impact on cell viability. Further advancements by researchers have optimized freezing techniques for carp sperm, enhancing post-thaw motility and fertilization rates. The Fish Sperm Cryobank at the IPC&C is among the earliest established worldwide (2). The preserved samples represent a valuable resource not only for the restoration of rare and economically significant species, but also for advancing fundamental research in cryobiological

processes. We are committed to fostering international collaboration and undertaking joint scientific investigations.

REFERENCES

1. Kopeika E et al (2019) *Probl Cryob Cryomed* **29**, 3-18. https://doi.org/10.15407/cryo29.01.003
2. Cabrita, E et al (2010) *J Appl Ichthyol* **26**, 623-635. https://doi.org/10.1111/j.1439-0426.2010.01556.x

PHYSIOLOGICAL HYPOXIA AS A REGULATOR OF SPERM CAPACITATION AND CRYORESISTANCE

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ABSTRACT

The success of in vitro fertilization relies on the functional quality of both fresh and cryopreserved spermatozoa, particularly their motility, viability, and fertilizing capacity (1, 2). This study aimed to evaluate the effect of different oxygen concentrations (1%, 5%, and 21%) during in vitro capacitation on human sperm functional parameters and post-cryopreservation survival. Ejaculates from 30 healthy men of reproductive age (mean age: 29.8 ± 3.5 years) were included. All samples met the WHO (2010) criteria for normozoospermia. Spermatozoa were prepared using the swim-up method and incubated under three oxygen conditions: 1% O_2 (hypoxia), 5% O_2 (moderate hypoxia), and 21% O_2 (ambient air; control). The acrosome reaction (AR) was assessed using FITC-PSA staining. Cryopreservation was performed both before and after swim-up preparation. Sperm motility and viability were highest under 5% O_2 conditions, indicating that this oxygen level provides optimal conditions for capacitation. The 5% O_2 group also exhibited the highest AR rate. In contrast, both motility and AR were significantly reduced in the 21% O_2 group ($p < 0.05$). Exposure to severe hypoxia (1% O_2) did not enhance motility or the proportion of capacitated sperm. Interestingly, non-capacitated spermatozoa exhibited greater cryoresistance compared to their capacitated counterparts ($p < 0.05$). Among the capacitated, cryopreserved samples, the highest post-thaw survival was observed in the 1% O_2 group. In conclusion, the oxygen level during incubation represents a critical determinant for sperm cryopreservation outcomes. Low-oxygen conditions provide distinct advantages: incubation at 5% O_2 promotes capacitation, hyperactivity, and the acrosome reaction, while 1% O_2 enhances membrane integrity and improves post-thaw survival.

REFERENCES

1. Petrushko M, Yurchuk T, Todorov P, Hristova E, Piniaiev V, Isachenko E, Rahimi G, Mallmann P & Isachenko V (2021) *Cryobiology* **103**, 39-44. https://doi.org/10.1016/j.cryobiol.2021.09.013
2. Petrushko M, Yurchuk T, Piniaiev V, Isachenko E, Rahimi G, Mallmann P & Isachenko V (2024) *Biopreserv Biobank* **22**, 597-599. https://doi/org/10.1089/bio.2023.0032

IMPACT OF ENVIRONMENTAL FACTORS ON SPERM QUALITY AND CRYORESISTANCE IN FISH: ANALYTICAL REVIEW

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ABSTRACT

Climate change, ecosystem degradation, and habitat loss are driving irreversible declines in the genetic diversity of fish populations. Cryobanking of reproductive cells offers a promising strategy for safeguarding aquatic biodiversity and supporting long-term conservation and restoration programs (1,2). This study aimed to identify key physical, chemical, and biological parameters of the aquatic environment that influence the morphological integrity and functional competence of fish spermatozoa in the context of cryopreservation. A comparative analysis of peer-reviewed publications, international environmental programs, and practical experience in gamete cryopreservation was performed, focusing on Carassius spp. Sperm quality and cryoresistance were evaluated in relation to pH fluctuations, temperature changes, dissolved oxygen variability, petroleum contamination, and ionic composition. Short-term exposure of Carassius auratus sperm to acidic water (pH 6.5) markedly reduced motility and viability prior to freezing, with literature evidence confirming that acidic stress destabilizes plasma membranes and increases susceptibility to cryo-induced lipid peroxidation, lowering post-thaw motility and fertilization rates. Petroleum pollutants induced mitochondrial dysfunction and ATP depletion, while elevated temperatures prior to sperm collection promoted reactive oxygen species formation, damaging membranes and DNA. Dissolved oxygen fluctuations and ionic imbalances (Na^+, K^+, Ca^{2+}) were further linked to oxidative stress, osmotic imbalance, and impaired enzymatic activity, collectively reducing post-thaw survival. These findings highlight that environmental stressor act synergistically, exacerbating sperm vulnerability to cryoinjury. Understanding these mechanisms is critical for developing adaptive cryopreservation protocols that reflect real-world aquatic conditions. Establishing optimized cryopreservation strategies, integrated with gene banking and environmental management, is essential for preserving the reproductive potential and genetic resources of vulnerable and commercially important fish species in the face of accelerating anthropogenic pressures.

REFERENCES

1. Kopeika E et al (2019) *Probl Cryobiol Cryomed* **29**, 3-18. https://doi.org/10.15407/cryo29.01.003
2. Li P, Li, Z.-H, Dzyuba B, Hulak M, Rodina M & Linhart O (2010) *Biol Rep* **83** 852–858. https://doi.org/10.1095/biolreprod.110.085852

A CALL FOR HARMONIZED TERMINOLOGY IN CRYOPRESERVATION: SUPPORTING REPRODUCIBILITY, GOVERNANCE AND COMMERCIALIZATION

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ABSTRACT

With cryopreservation technologies advancing rapidly, transforming the capacity to preserve biological materials such as cells, tissues, organs for transplantation, and even whole organisms, harmonized terminology is essential to support scientific progress, regulatory approvals, and commercialization. Yet because cryobiology research spans diverse disciplines including engineering, biology, chemistry, physics, medicine, and conservation, the convergence of these disciplines has led to discordant terminology, reflected as inconsistencies in the broader cryopreservation literature. As cryopreservation applications continue to expand with broad societal impacts in medicine, food sustainability, and conservation, harmonizing terminology is increasingly urgent. In biomedicine at small-scales, these applications include cryomesh-enabled vitrification of drosophila embryos, preservation of pancreatic islets, and metal-forms-enabled nanowarming of arteries and heart valves. Larger-scale successes include subzero preservation and transplantation of pig kidneys after transatlantic transport, partial freezing enabled transplant of pig kidneys, transplantation of a hypothermic preserved human heart aboard a commercial flight; multi-day storage of supercooled human kidneys and livers; and life-sustaining transplantations of small animal (rat and rabbit) vitrified kidneys enabled by dielectric and nanowarming methods. Examples of applications in biodiversity conservation include isochoric vitrification of coral fragments, laser nanowarming enabled preservation of coral larvae, etc. In agriculture and aquaculture, proof-of-concept has been shown in the isochoric freezing of vegetables (potatoes) and fruits (apples and cherries), and in laser rewarming of vitrified shrimp (nauplius-V stage) larvae. We propose a call to action urging key organizations, such as the Society for Low Temperature Biology, to lead a consensus-building effort to standardize terminology for research, application, and policy making. We will present examples of inconsistent cryopreservation terminology; compare research domains where inconsistent terminology has led to scientific, ethical, legal, and social problems; and suggest a process to achieve harmonization. The Society for Low Temperature Biology being a key stakeholder, its annual meeting offers an ideal forum to present this call to action.