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CRYOBANKING OF VEGETATIVELY PROPAGATED PLANTS – FIFTEEN YEARS OF EXPERIENCE

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Cryopreservation is a method for long-term storage of living organisms or of their parts at ultra-low temperature. It allows conservation of plant germplasm of vegetatively propagated plant with low maintenance costs and without the risk of biotic and abiotic stresses or the danger of their genetic change (1). In the Czech Republic, there are several significant collections of plant germplasms: garlic, hops, grapevine, potatoes and fruit trees that are vegetatively stored and propagated. In order to improve the standards and safety of their storage the Cryobank of vegetatively propagated plants was establish at the Crop Research Institute in Prague in 2003. Progress in cryopreservation method and the cryobank improvement has been made since 2005 due to close European collaboration among plant cryobiologists as a result of the CRYMCEPT project and subsequent the COST Action 871 initiative (2). This collaboration resulted in the EURALLIVEG (3), the pilot European project on cryoconservation of European garlic collections (2007-2011). Selected garlic genotypes have been reciprocally stored in the tripartite garlic cryobank (Czech, German and Polish). The use of thermal analysis for the development of cryopreservation methods has been considered as a very important milestone for the improvement of cryopreservation. Cooperation with experts in the field of theoretical and applied physics and physical chemistry was essential for mastering these techniques and their application in plant cryopreservation. This experience was shared by the COST Action 871 Thermal analysis training school (2007-2009). Cooperation has recently begun on the cryopreservation of forest trees and microorganisms with the Forestry and Hunting Research Institute and the National Program for the conservation of major microorganisms in agriculture, respectively.

Acknowledgements: This work was supported by QJ1630301 and RO0418 projects of the Ministry of Agriculture of the Czech Republic.

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CRYOPRESERVATION AND BIOBANKING OF HUMAN ORGANOIDS: CURRENT STATUS AND FUTURE PERSPECTIVES

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Organoids represent in vitro grown 3D cell aggregates derived from stem cells or primary tissue. They are capable of self-renewal, self-organized differentiation and display organ functionality. Because organoids have similar composition and architecture to primary tissue, they represent a relevant experimental model of *in vivo* conditions. To this date, organoids have been used extensively used for analysing stem cell behaviour, identifying niche components, modelling pathogen–epithelia interactions, gene editing, and disease modelling. This experimental success has enhanced efforts to create cryopreserved biobanks of both, healthy and diseased, human organoids as a renewable resource that is accessible to researchers worldwide. This presentation will summarize current status and future perspectives of human organoid research, efforts of organoid cryopreservation and their biobanking.

SUB NORMOTHERMIC UNFROZEN STORAGE AND SHIPMENT OF CELLS AND TISSUES FOR THERAPY: REPORT FROM A WORKSHOP HELD AT UNIVERSITY COLLEGE LONDON MAY 2018

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The difficulty in developing effective cryopreservation methods means that storage and shipment of cells in an unfrozen state at temperatures below typical culture conditions is often the means of cell supply considered by those shipping cells for therapy and diagnostic purposes. However, such storage could impact on the stability and suitability of cells for clinical use or diagnosis. A workshop of experts was organized (9th May at University College London) on the “sub normothermic unfrozen storage and shipment of cells and tissues”. A number of key regulatory issues were raised including lack of standardized analytical methodologies; the potential need for pre-use evaluation after the cell product had been released and shipped for use, and the significant challenge of introducing new storage and shipment conditions in the absence of historic validation data. Reduced temperature and oxygen tend to inhibit metabolic rate and were identified as key factors in enabling stability of viability and function during storage. However, it was concluded that there were a wide range of biological effects in the 0-4^{o}C range even in simple cellular systems which include cold-shock protein induction, formation of membrane holes and waxy lipids in membranes. Even at higher temperatures changes such as surface molecule crosslinking could affect product quality. A number of commercial reagents and new hydrogels were identified which had been applied successfully to store certain cell types. However, storage solutions were often derived empirically and published data often used diverse viability assessment methods which made comparison of results with the different reagents very difficult. Key requirements identified for future research outputs were enhanced viability assays supported by a better understanding of fundamental biochemistry and investigation of small molecules which may have beneficial effects for cell shipment.

IMPACT OF CHANGES OF THE EUROPEAN UNION LEGISLATION ON CRYOPRESERVATION TECHNOLOGIES – EXPERIENCE OF THE TISSUE BANK UNIVERSITY HOSPITAL HRADEC KRALOVE

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Aim: To review own experience with the impact of the EU Directives regulating safety and quality of cells and tissues used for clinical transplantation as well as of regulation of advanced cell therapy products on practice of the tissue bank (TB) and cell therapy laboratory. **Methods:** The main tool used for achieving compliance with Directives 2004/23/EC, 2006/17/EC, 2006/86/EC was complete reconstruction of the TB. It made possible to prevent secondary contamination during cell and tissue processing by using clean room technology –critical processing area of the Grade A with the background of the Grade B. Cross contamination during storage at liquid nitrogen temperatures was prevented by double bagging of haematopoietic cell concentrates or solid tissues, e.g. vascular grafts (3) as well as by using exceptionally vapour phase of liquid nitrogen for storage. Safe storage was possible due to use of large liquid nitrogen containers with automatic filling and continuous registration of temperature and liquid nitrogen level. The harmonization process was completed by introducing European Coding System (ISBT 128, where applicable) in 2017. **Results:** In the year 2004 the TB was granted provisional multi-tissue licence by the Ministry of Health, in 2011 it received the licence issued by the State Institute for Drug Control. In 2017 it was given the European Union Tissue Establishment Codes CZ 000425 for reproductive tissues, CZ 000426 for haematopoietic tissues and CZ 000427 for other tissues, e.g. vascular grafts. In 2011 approval for manufacturing advanced medicinal products was granted. As the result it was possible to perform cryopreservation of cultured mesenchymal stromal cells (producer BioInova Ltd., Prague) as an experimental part of approved clinical trial. **Conclusions:** In cryopreservation of minimally manipulated tissues the TB was able to achieve compliance with the Directives. Cryopreservation of advanced medicinal products is still in the experimental stage.
Acknowledgements: Cryopreservation of advanced medicinal products was supported by the grant No. TG02010020.
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CRYOPRESERVATION IN TISSUE MEDICINE: THEORY AND PRACTICE

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Cryopreservation plays a major role in the storage of tissues that are kept for subsequent transplantation. A wide variety of tissues intended for transplantation for a patient are stored in this way. In addition to the assignment to a suitable recipient, it must be ensured in particular that a sufficiently long period of time is available for infection diagnostic analyses. Cryopreservation of tissue is therefore routine in everyday tissue medicine. As is well known, the success of low temperature storage depends in particular on coordinated freezing and storage processes, in the optimization of which many research approaches have already been invested. In contrast to these well-known principles of important parameter adjustment in this context, little attention is paid to optimizing the freezing process in the daily routine of tissue medicine. In fact, for some applications the suboptimal storage of tissue is not critical, as no vital cells are necessary or desired. This reduces the process to a minimum and eliminates the need for additives such as cryoprotective agent (CPA) or animal/human serum. This is particularly important for tissue preparations which, as in Germany, require a medicinal product approval by a competent authority. Any change to the process must be first approved by the competent authority, the Paul-Ehrlich-Institut (PEI). Optimizing the freezing process would therefore entail a new approval procedure. If the changed process was not previously known in the EU, a clinical study is also required. For this reason, the conditions are not improved in case of doubt, although it is known that this could lead to a higher quality of the tissue after thawing. Less toxic CPA could simplify the approval procedure and thus contribute to improved tissue storage for transplantation.

OVARIAN TISSUE CRYOPRESERVATION: PAST, PRESENT AND FUTURE DIRECTIONS

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While still considered an experimental procedure in most countries, ovarian tissue cryopreservation has been increasingly applied worldwide to restore fertility in patients with malignant and non-malignant pathologies with risk of premature ovarian insufficiency. This exponential upsurge in the number of ovarian tissue cryopreservation procedures in Europe in the last years, being comparable to the number of oocyte cryopreservation for cancer patients, is mainly due to the successful results after transplantation of cryopreserved ovarian tissue. Indeed, this strategy had led to restoration of ovarian function of ~95% of the patients and more than 130 live births. As promising as these outcomes are, procedures to cryopreserve human ovarian tissue have been empirically created based on protocols for embryos, oocytes or ovarian tissue from other animal species. Probably due to their unspecific nature, these procedures have showed to damage ovarian tissue and negatively affect follicle populations. This lecture aims to summarize the current ovarian tissue cryopreservation procedures and different approaches that have been tested to improve graft outcome and it will discuss other applications for this strategy.

CRYOPRESERVATION OF IPSC-DERIVED MEGAKARYOCYTES

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A wide range of clinical conditions, including acute blood loss, sepsis, genetic abnormalities, consequences of oncology treatment, and other, may results in severe life-threatening thrombocytopenia. Platelet transfusion is virtually the only effective therapeutic approach providing successful

thrombocytopenia treatment. However, platelet transfusion is often complicated by immunological issues, costly and intricate donation procedures, insufficient availability of supply, quality and contamination issues, as well as short shelf life of donated platelets. Thus, to overcome these issues, the research is focused on possibilities of generating populations of functional platelets and megakaryocytes (MKs) ex vivo. Current studies report the capability of iPSC-derived MKs to release functional platelets in circulation after in vivo transfusion in the animal model and even in phase I clinical trials (1-4). Additionally, iPSC technologies may allow generating patient specific, as well as "universal" cell types addressing the immunology issues (1). Due to the challenges with donated platelets, application of iPSC-derived MKs opens perspectives as an alternative or additional approach to conventional donor platelet transfusion. At the same time, majority of pathological cases requiring such transfusion necessitate immediate availability of these cells in sufficient amounts and functional form. Development of efficient cryopreservation procedures would provide the opportunity to long-term storage and accumulation of necessary amounts of MKs. However, in this case, besides the viability it is crucial to consider the recovery of functional parameters after the impact of subzero temperatures. In this study we explore the possibility to cryopreserve iPSC-derived MKs with application of various cryoprotectants. The MKs were analysed for phenotypic and functional parameters before and after cryopreservation. We were able to recover over 80% of cells after cryopreservation with Me2SO and propylene glycol. Survived cell populations possessed typical MK phenotype and functionality statistically similar to the cells before cryopreservation. Additionally, possibility of transfusion of iPSC-derived MKs after cryopreservation was tested in animal model in vivo.

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STEPPED VITRIFICATION PROCEDURE TO CRYOPRESERVE HUMAN OVARIAN TISSUE: WE ARE NOT THERE YET

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Liquidus tracking method consists in a stepped vitrification procedure that has been successfully applied in different types of tissues. The main goal of this approach is to promote vitrification, avoiding ice crystal nucleation, while decreasing the toxic effects of high concentrations of cryoprotectants. In order to test this method for human ovarian tissue cryopreservation, ovarian cortex from 7 adult women were used. Samples were submitted to the stepped vitrification protocol previously developed for bovine ovarian tissue (1). In this protocol, samples were transferred towards higher concentrations of DMSO with subsequent cooling above the freezing point. Cryosubstitution showed that our protocol successfully avoided the formation of ice crystals in the samples. X-ray computed tomography was used to investigate DMSO concentration in the tissue after vitrification procedure and DMSO removal. This analysis revealed that vitrified samples had an average DMSO concentration of 42 ± 4% and our cryoprotectant removal step was able to completely remove DMSO from the tissue samples (0.06 ± 0.75%). After warming and 24h *in vitro* culture, ovarian tissue fragments were fixed and analyzed. Histological evaluation showed a massive degeneration in the ovarian stroma and follicles. Moreover, ultrastructural analysis showed

mitochondria degeneration, alterations in chromatin condensation, cell vacuolization and extracellular matrix swelling in both, stroma and follicular cells, which are typical alterations signs of DMSO toxicity. In conclusion, while our protocol prevented ice formation by ensuring a proper DMSO concentration in the ovarian tissue, our findings suggest that such high cryoprotectant concentration was toxic for human ovarian tissue. More studies are necessary to calculate the maximum DMSO concentration that should be used in our stepped vitrification procedure in order to preserve ovarian stroma and follicles.
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NEW PERSPECTIVES OF OVARIAN TISSUE CRYOPRESERVATION BY MEANS OF X-RAY COMPUTED TOMOGRAPHY

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Thanks to the most recent advances in cancer treatments the number of cancer survivors is progressively increasing. However, these treatments have gonadotoxic effects which cause infertility in most cases. Cryopreservation of ovarian tissue is, often, the only option to preserve the fertility for oncological patients, especially in the case of prepubertal girls (1, 2). We have used the technology of X-ray Computed Tomography to analyze human and bovine ovarian tissue after cryopreservation and rewarming in order to evaluate the cryoprotectant (CPA) concentration and distribution within tissues. We have selected Me2SO as CPA, since the sulfur atom of the molecule makes the X-ray attenuation proportional to its concentration (3). On the one hand, we have assessed the conventional freezing protocol, the most common method used for ovarian tissue cryopreservation so far, by studying the influence of different parameters of the protocol (4). On the other hand, herein we present the results obtained for the development of an alternative procedure for ovarian tissue cryopreservation: a vitrification method performed by steps (5). This method consists of increasing the CPA concentration to allow the sample to vitrify, avoiding the crystalline structure of ice, while decreasing the temperature to reduce the toxicity effects of those high CPA concentrations. In order to deal with the permeation issues, we designed a controlled system that keeps the samples in a continuous agitation. Results show very interesting aspects regarding the conventional freezing protocol, such as the uneven CPA distribution achieved in tissues after cryopreservation or the role of the seeding temperature. Additionally, new perspectives of ovarian tissue cryopreservation are opened in the field of vitrification methods. Nevertheless, there is a concern about the tolerance of ovarian tissues to high CPA concentrations and further investigation should be addressed to this point to achieve accurate alternative methods.
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DNA METHYLATION CHANGES AFTER CRYOPRESERVATION: MSAP ANALYSIS BY MULTIVARIANT STATISTICAL METHODS

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Cryopreservation allows long-term conservation, and the genetic stability of the preserved material is one of its most important features compared to other conservation techniques. However, this stability has been questioned and in some cases epi-genetic instability after cryopreservation has been detected. Epigenetic information includes heritable signals that modulate gene expression but are not encoded in the primary nucleotide sequence. DNA methylation is the most extensively studied epigenetic modification and it has been connected to many crucial biological processes. The central issue of this work was to detect epigenetic changes in the cytosine methylation pattern throughout the whole cryopreservation process in an encapsulation-dehydration protocol in *Mentha xpiperita* apices. Although many techniques have been described for analyzing DNA methylation, MSAP (methylation sensitive amplified polymorphism) is widely used. The global DNA methylation status has been studied after each step of the cryopreservation protocol. The effect of the addition during the procedure of vitamin E or ascorbic acid was also evaluated. All the methylation variations detected were statistically analyzed via multinomial logistic regression in order to test their significance. This study intends to introduce a new statistical approach to address methylation data in this context. The results obtained showed and statistically proved that significant differences in the methylation pattern existed among the steps of the protocol.
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TWENTY YEARS OF CRYORESEARCH ON PINEAPPLE SHOOT TIPS IN CUBA

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In Cuba the research aimed at the conservation of plant genetic resources for food and agriculture has received special attention and has benefited from support through international and national technical cooperation projects, which have fostered the training of human resources and creation of technological infrastructures in different institutions of the country. In the case of the of pineapple crop, the application of cryopreservation techniques for shoot tips of *in vitro* plants has been investigated for more than 20 years. Shoot tips cryopreservation is the most appropriate strategy for long-term storage of the genetic resources of vegetatively propagated species. In our work we presented the studies performed by IPGRI Project to set up and refine a cryopreservation protocol for apices of pineapple *in vitro* plantlets. The protocol established following the vitrification approach was successfully applied for the first time in 1998 to shoot tips of three pineapple varieties, and then extended to nine pineapple accessions belonging to the *in vitro* collection of Bioplantas Centre in Cuba in 2005. Moreover, five years ago by multidisciplinary co-operation between Cuban and Brazilian Institution (EMBRAPA) was developed of cryo-technology using droplet-vitrification procedure of pineapple shoot tips including cryobionomic studies and smallholder producers' participation. For example: Positive effect of application of Jasmonic acid during cryogenic technology. Influences the morphological indicators (plant length, the length of the largest leaf and the diameter of the base) of the starting plant material. Influences the morpho-physiological indicators (fresh weight, chlorophyll a, b, and total, net photosynthesis and water use efficiency) of shoots regenerated after the development of protocol for cryopreservation. Innovative research on pineapple cryobionomics was

carried out (e.g. histological, biochemical, molecular, ex vitro hardening under greenhouse and field conditions). Recently, in collaboration with Japan at NIAS was set up the preliminary protocol using cryoplate technology for pineapple shoot tips.

THE CRITICAL THERMAL RANGE FOR CONTROLLED RATE COOLING FOR MAMMALIAN CELL CRYOPRESERVATION

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Slow, controlled cooling is essential for successful cryopreservation of most somatic mammalian cells. In practice, samples are typically transferred to long-term cryogenic storage once the controlled-rate cooling process has reached an endpoint of around -80°C to -100°C. Placing samples in the liquid or vapor phase of nitrogen too early could result in rapid cooling rates, leading to intracellular ice formation and cell death. However, there is little basis in the literature for the controlled cooling endpoint to be set at -80°C or below. The present work explored the critical temperature for the endpoint of the controlled-cooling phase in four different mammalian cell lines (Jurkat, HepG2, MG63 and CHO cells) through a combination of biological and physical measurements. Cryovials with 10^6 cells, 1 ml fill and 10% DMSO as cryoprotectant were cooled at a 1°C/min in a VIA Freeze controlled-rate freezer, and samples were transferred to liquid nitrogen at different endpoint temperatures (4°C, -10°C, -25°C, -40°C, -50°C, -60°C, -80°C and -100°C). Cell viability, proliferation and functionality measurements post-thaw showed highest and comparable values for transfer temperatures of -50°C and below for the four cell lines tested. Transfers of samples performed at higher temperatures ≥(-40°C) resulted in a drastic loss of viable and functional cells, while Differential Scanning Calorimetry (DSC) measurements preformed on Jurkat cells revealed an intracellular glass transition temperature (T_{gi}) of -46.9 ± 1.3°C.These results suggest that controlling cooling until T_{gi} is reached is critical for a successful cryopreservation of a wide variety of mammalian systems, and no biological advantage is conferred by further cooling. Shorter controlled-rate cooling cycles could thus safely be applied, saving time and potentially allowing more cryopreservation cycles to be completed a day.

RETROSPECTIVE ANALYSIS OF DIMETHYLSULPHOXIDE LOAD IN AUTOLOGOUS PERIPHERAL PROGENITOR CELL TRANSPLANTATION IN MULTIPLE MYELOMA

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Aim: To perform risk assessment of infusion amounts of Me2SO higher than maximal Pharmacopoeial daily dose 1g/kg during autologous PBPC transplantations in patients suffering from multiple myeloma. **Methods:** The total of 30 men and 30 women in the age of 38 -72 years transplanted at the IV.

Department of Internal Medicine of the University Hospital Hradec Kralove in the years 2016-2018 were included in the study. Cryopreservation was performed in 100 ml units by controlled rate freezing in presence of 10% (V/V) Me2SO in the licensed Tissue Establishment: EUTE CODE CZ000426. The units were stored at liquid nitrogen vapour phase. At the moment of infusion of thawed concentrates following parameters were evaluated: Total volume of infused PBPC concentrate, dose of CD 34+ cells/per kg, volume and/or weight of Me2SO per kg. **Results:** In the group of men the amount of infused Me2SO ranged between 0.1 and 0.96 g/kg, SD 0.21, Median 0.28 in the group of women between 0.27 and 1.7 g/kg, SD 0.31, Median 4.8. The dose higher than 1g/kg (1.7/kg) was found in one case only (1.7%). In this case transplantation was performed within two days. The mean CD 34+ cell dose/kg was 6.75, SD 9.4 in men and 5.3, SD 2.04 in women. **Conclusions:** Our experience shows that the risk of loading multiple myeloma patients with Me2SO doses higher than prescribed by European Pharmacopoiea per day is low.

CRYORESISTANCE OF POLAR EUKARYOTIC MICROALGAE

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Environment in Polar Regions is characterized by many extremes. Low temperatures, lack of fluid water, irregular nutrient and light supply, fluctuations in daily and annual cycles could seem unfavourable for life. In spite of this, many groups of phototrophic microorganisms adapted well to and dominate in a wide range of polar habitats. To successfully colonize low-temperature environments, photoautotrophic microorganisms have evolved a various strategies that span from molecular to whole cell and ecosystem levels. Under freezing conditions it is very important for phototrophic microorganisms to maintain an aqueous external environment. Therefore microalgae are able to produce many extracellular macromolecular substances together with changes of physiological pathways that can help to provide such environment. Also resting stages formation is reported in many groups of phototrophic organisms including cyanobacteria and green algae. According to these natural conditions and risks microalgal communities from Polar Regions should be more resistant to freezing injuries. Several field studies of cyanobacteria and algae showed a habitat dependency. For example, strains isolated from seepages were less tolerant to desiccation and freezing than those from other wetland habitats. Likewise a higher tolerance of terrestrial diatoms to temperature extremes was indicated. In a comparison focused on temperate benthic diatoms, only terrestrial species persisted experimental freezing. This is in agreement with the high population differentiation observed in a freshwater benthic diatoms and may explain their dispersal capacities and widespread endemism. On the other hand some experimental studies show sensitivity of some groups of microalgae to experimental freezing suggesting it is likely that differences among algal strains are determined by the specific characteristics of each strain (species) rather than the habitat or original locality as demonstrated on the ability of both polar and temperate strains. Better understanding of molecular, physiological and ecological principles of cryoinjuries resistance of microalgae including multiple stress adaptations to polar environments could help us to develop cryobiology knowledge and cryopreservation techniques.

POLLEN GRAINS AS BIOGENIC SOURCES OF ICE NUCLEI - A REVIEW

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David Sands proposed the concept of rain-making bacteria (*Pseudomonas syringae*), commonly referred to as bioprecipitation, in the 1980s (1). Since then, other biogenic sources of ice nuclei in clouds have been identified such as fungal spores and pollen grains (2). Recent technological developments in bioaerosol research at the immersion freezing mode facilitate the detailed analysis of ice nucleation phenomena (3-6). The results of these analyses will be discussed as well as their potential environmental and biotechnological applications.

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CRYOPRESERVATION OF DORMANT BUDS OF FRUIT TREES

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The safe and efficient fruit trees germplasm conservation is important for the future breeding programme as well as for maintaining the broad spectrum of biodiversity of germplasm collections. The aim of the work is to compare different fruit species dormant bud cryopreservation results. The cryopreservation of dormant apple, pear, apricot and other fruit species was evaluated. The results suggest that the dormant bud cryopreservation technique offers a safe and valuable system for back up the field and *in vitro* fruit trees germplasm collections.

APPLICATION OF INFRARED VIDEO THERMOGRAPHY FOR MONITORING OF FREEZING/THAWING EVENTS WITHIN 3D COLLAGEN-HYDROXYAPATITE SCAFFOLDS

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Preparation of tissue-engineered products with controlled cryostorage properties is an innovative multidisciplinary approach for the translation of regenerative medicine research from bench to clinic. Thus, hydroxyapatite nanoparticles in scaffolds not only increase their mechanical properties but could also serve as a source of numerous sites providing uniform crystallization and temperature distribution upon freezing and thawing. In this work, we address the potential benefits of using infrared video thermography for monitoring freezing/thawing events in 3D porous collagen-hydroxyapatite scaffolds. The scaffolds were prepared by freeze-drying of mineralized collagen suspensions and characterized by Raman microscopy and X-ray microcomputed tomography. Scaffold biocompatibility with mesenchymal

stromal cells from common marmoset monkey *Callithrix jacchus* was investigated using SEM and fluorescence microscopy. It was shown that scaffolds containing hydroxyapatite have lower heat capacity than that of without hydroxyapatite. Infrared video thermography provided effective visualization of freezing/thawing events within scaffolds frozen 'in air' and in solution in different cell culture plates as well as in cryobags and proved to be a highly informative strategy. In addition, several scenarios of thawing were simulated in a model comparing heat transfer during thawing ('in air' and in solution) using finite element method and StarCCm+ software. The results obtained could serve as a basis for further development of protocols for efficacious cryopreservation of 3D tissue-engineered products.

CRYOPRESERVATION OF MICROORGANISMS

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A safe, long-term maintenance of viable microbial cultures is of paramount importance culture collections, biotechnology, etc. Cryopreservation of microorganisms (viruses, bacteria, microfungi, algae, protozoa) below -140°C (ensuring that any biochemical and cryogenic processes are stopped) is an optimal preservation technique that can be widely applied. It usually retains high viability as well as phenotypic and genomic stability of stored microorganisms which is crucial when the microbe is being used as a reference, type or production strain. There are several factors that affect cryoresistance of microorganisms, e.g. freezing menstruum, cryoprotective additives, cooling and warming rates, recovery medium, and they should be optimized.

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APPLICATION OF THERMAL ANALYSIS IN PLANT CRYOPRESERVATION

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Thermal analysis is crucial for successful cryopreservation. Mostly, a differential scanning calorimeter is used for thermal analysis. Using thermal analysis, we can determine the volume of frozen, unfrozen, unfreezable water and glass transition temperature in tissues or organs of plants. The volume of frozen/unfrozen water is the primary precondition for the survival of plants exposed to ultra-low temperatures. Less frozen water in plant tissue has greater chances of higher regeneration rates. The large volume of frozen water inside cells is lethal due to the crystal growth. Therefore, the plant parts are dehydrated before cryopreservation. Water is removed by various techniques such as dehydration by dry sterile air flow, by osmotic or frozen dehydration. Dehydration has its limits, with excessive dehydration, cell death of water scarce occurs. Thus, thermal analysis can show an optimal "window" of water content, where there is neither less nor much water in the cells for high regeneration after cryopreservation. Unfreezable water can also be determined by thermal analysis. Unfreezable water is defined as the volume of frozen water extrapolated to the total volume of water determined by drying in the oven. The role of unfreezable water in tissue has not yet been fully understood. The same principles and the determination of the thermal parameters can be used for cryopreservation of recalcitrant seeds use cryopreservation methods as for hydrated parts of vegetatively propagated plants. The temperature of individual peaks, corresponding, for example, to changes e.g. in phospholipids and proteins, can be determined by thermal

analysis for orthodox seeds. The same varieties of one species have very similar thermal fingerprints with peaks at the same temperature but differ in area and the ratio between them. Thermal analysis can be advantageously used to optimize the concentration, ratio and duration of cryoprotective agents.
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FINITE ELEMENTS COMPUTATION OF ORGAN REWARMING BY HIGH INTENSITY FOCUSED ULTRASOUNDS

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The heating by means of High Intensity Focused Ultrasounds of a vitrified organ, like a rabbit kidney, has been simulated by the COMSOL® software. COMSOL is a package of finite element analysis and resolution software that uses different specific application modules, allowing the coupling between them. For this case, the acoustic and heat transfer modules have been used, so that the acoustic module allows calculating the flat wave dissipation density generated by focused ultrasound in the frequency domain, and the module Heat transfer, from the data obtained in the acoustics module, solves how heat is transferred to the system over time. The aim of the simulation is to heat the organ homogeneously and quickly from cryogenic temperatures -140°C (133 K) to temperatures close to room temperature, in this case -20°C (253 K). After solving the corresponding sound propagation and heat transfer equation, for the set of transducers, a quick (100 K/min) and uniform rewarming of the organ is possible at realistic frequencies (1 MHz) and powers (50 Watts per transducer). The conclusion of this work is that it is possible to achieve enough quick and uniform rewarming of a small organ by means of high intensity focused ultrasounds, in order to avoid recrystallization for a concentration of cryoprotectant below the limit of the toxicity. This technique opens new paths towards tissue and organ banking.

SUB-NORMOTHERMIC STORAGE OF HEK293T CELLS USING A "NOVEL" BIOPRESERVATION MEDIUM, AQIX® RS-I

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Background: Cell based therapies are an exciting and rapidly developing technology. However, there are logistical problems related to cell storage and shipment. Recently, Coventry University and Life Science Group Ltd were awarded Knowledge Transfer Partnership (KTP) funding via Innovate UK to further develop AQIX® RS-I and its applications. AQIX® RS-I is a physiological solution based on human interstitial fluid, developed to maintain viability of isolated mammalian tissues and organs. Additionally, AQIX® RS-I may also have cell culture applications. As such, one of the areas that the KTP project is focusing on is the development of a novel, xeno-free medium suitable for sub-normothermic non-frozen cell storage. Being xeno-free maintains the principles of the 3R's by being free of animal products, such as foetal bovine serum (FBS). Additionally, development of a xeno-free storage medium will address logistical issues associated with cell-based therapies. *Methods*: HEK293T cells were plated at 1.5 x 10^6 cells per T25 in 5 ml complete DMEM + 10% FBS with antibiotic-antimycotic and were cultured at 37°C, 5% CO_2 for 24 h. Media was replaced with the storage medium; DMEM/HEPES or AQIX® RS-I. Cells were then stored at ambient temperature, in darkness for 96 h. To recover the cells, the storage medium was replaced with complete DMEM + 10% FBS with antibiotic-antimycotic and cells were cultured at

37°C in 5% CO_2 for 72 h. Cells were then dissociated and viability was assessed using trypan blue. *Results*: Following 72 h recovery, cells stored in AQIX® RS-I showed a 2.3 fold increase in cell number compared to the number of cells seeded and were 99% viable. Whereas, half of the original number of cells remained, following storage in DMEM/HEPES with only 73% viability. *Conclusion*: Preliminary work indicates that following 96 h storage in AQIX® RS-I at ambient temperatures, HEK293T cells recover well and demonstrate excellent viability and proliferation. These initial results are promising, and further research will evidence that AQIX® RS-I is an optimal base on which to develop a transport and storage medium suitable for cell-based applications.

MICRO- AND LARGE VOLUMES CRYOPRESERVATION OF SINGLE HUMAN SPERMATOZOA USING NON-PENETRATING CRYOPROTECTANT

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Cryopreservation of spermatozoa is an integral part of assisted reproductive technologies (ART) used in the treatment of patients with a male factor of infertility. Traditional methods of spermatozoa cryopreservation are not optimal in the case of patients with pathozoospermia. Cryopreservation of large volume of sample with the using of penetrating cryoprotectants requires washing which leads to decrease in the quality, quantity and motility of spermatozoa (1, 2). *Aim* of work was to assess kinetic characteristics of spermatozoa from pathozoospermic ejaculates after micro- and large volumes cryopreservation using non-penetrating cryoprotectant. *Materials and methods*. Spermatozoa were obtained from ejaculate of patients with pathozoospermia who underwent treatment of infertility by ART. Sperm preparation was done through density-gradient separation performed according to Sydney IVF, Cook Medical Manual followed by swim-up procedure. Cryoprotectant medium consisted of 0.5M sucrose (Sigma, USA) and 20% human serum albumin HSA (LifeGlobal, USA) in a Sperm Preparation Medium (Cook Medical, USA) was added in an equal volume of sample. Micro-volume of spermatozoa (10 µl) in micro-straw and large volume (500 µl) in cryovials after 10 min incubation with cryoprotective media were immediately plunged into liquid nitrogen. After thawing using the water bath the kinetic characteristics of the spermatozoa in each sample were evaluated. *Results*. After freeze/thawing 64 ± 2.9% of spermatozoa in micro-volume straw remained their motility. This index was 47 ± 3.3% for spermatozoa cryopreserved in cryovials. Freezing spermatozoa in micro-volume straws allows to be used it promptly after thawing without washing step. It can improve the ICSI procedure and optimize cryobanking since it reduces the costs of sample storage.
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COMPLEX DESCRIPTION OF CRYOPRESERVED CELL NUCLEI DEFECTS BY IMMUNOFLUORESCENCE MICROSCOPY: DNA LESIONS, CHROMATIN DECONDENSATION, NUCLEAR MEMBRANE RUPTURES

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The factors contributing to cell protection from freezing injury are still not completely elucidated. Many studies have addressed cryopreserved cell survival but have not correlated it with the impact of the freeze/thaw process on the state of cell nucleus. In this work, we observed changes in cell nuclei (DNA, chromatin, nuclear membrane) and related them to parameters of a slow-freezing protocol of NHDF cells. Namely, we investigated the consequences of freezing/thawing in terms of DNA double-strand break formation, higher-order chromatin structure alteration and nuclear envelope disruption. Moreover, we analysed the relationship between these structural parameters and the use of DMSO, trehalose and antifreeze protein ApAFP752 as respective cryoprotectants. Our results contribute to the deeper understanding of the freezing processes and their impact on the cell nucleus, which then offers clues for rational design of new cryofunctional materials and cryoprotective protocols.

CRYOPRESERVATION OF CZECH HOP GERMPLASM

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Hops breeding, growing and utilization has been traditional and important in the Czech Republic from the middle age. This crop is propagated and maintained vegetatively. Nowadays hop collection includes 400 accessions placed at the Hop Research institute Co., Ltd. (HRl) in Zatec in field conditions but they are endangered by biotic and abiotic stressors. Conservation of hop germplasm in the field collections increases the risk of accidental loss of valuable genotypes. The method of cryopreservation reduces this risk and allows safe storage of the plant samples at ultra-low temperatures. This method contributes to the conservation of genetic stability and prevent from ageing. It is used for conservation of genetic resources of cultural and wild plants in such virus free material, which is endangered by biotic and abiotic stresses if multiplied in field conditions. Development of cryo-collection is the best way to eliminate the risk of an accession lost (1). *In vitro* cultures were derived from isolated meristems tips according to procedure described by Svoboda (2) and maintained by Svoboda (3) and Faltus et al. (4). Simple cryopreservation method was used for cryopreservation of selected genotypes of the Czech hop germplasm collection. Nodal cutting were acclimated by low temperature and sucrose treatment. Isolated shoot tips were loaded with 0.7M sucrose for overnight and subsequently dehydrated above silicagel for approximately 100 minutes on aluminium plates. Shoot tips were plunged directly into liquid nitrogen. Control explants were thawed at 40°C water bath and regenerated on medium for 8 weeks. Altogether 45 hop genotypes have been cryopreserved with average recovery rate of 40%. 79% of accession showed higher plant recovery than 30%. The minimal number of plants to recover for each cultivar was calculated as a sum of minimal numbers of viable plants in particular cryopreservation procedures according to a probability tool developed by Dussert et al. (5). The methods used and results are presented.
Acknowledgements: This study was supported by the research project QJ1630301 of the Ministry of Agriculture of the Czech Republic.
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PRESERVING BACTERIA WITH OLIGOSACCHARIDES AND ECO-FRIENDLY PROCESSES (PREMIUM)

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Many microorganisms' potential remains unexploited due to the current inability to preserve them at an industrial scale. Lactic acid bacteria (LAB) are a group of microorganisms widely used for producing a wide diversity of fermented foods. The market of concentrated cultures (starters) is continuously growing due to the development of health benefits products, the use of plant origin proteins as fermentation substrate (instead of milk proteins) and to LAB's ability to convert agricultural by-products (green chemistry). The manufacture of starters requires the application of successive operations that generate stresses, including storage and delivery, as well as reactivation (thawing, rehydration). These operations usually lead to cellular damage and loss of functionalities, in particular following the stabilisation processes: freezing, freeze-drying, spray drying. In addition, these processes generate environmental impacts, due to energy consumptions and use of the cold chain. Completely revisiting the process of LAB preservation thus needs to be undertaken, integrating all the steps and the three dimensions involved: product quality, process efficiency and environmental impact, to develop original and innovative alternatives to companies and society. In this context, a four-year multidisciplinary project funded by the European Commission, PREMIUM project, involving academic and industrial partners from France, Argentina, Portugal, Spain and the United Kingdom, proposes to develop new strategies to preserve LAB from laboratory to industrial scale. Using multi-criteria analysis will identify the most promising strategies for industrial eco-friendly preservation of micro-organisms. Moreover, after validation from lab to industrial scale on a small number of strains, other micro-organisms and mammalian cells will be tested. The project will thus pave the way for future commercial exploitation of new protective formulations (composed of oligosaccharides), by extending to other cells and by drawing up a feasibility study for spin-off activities regarding the sourcing of new protective molecules to the standards required for clinical application.

COMPARING DROPLET-VITRIFICATION AND ENCAPSULATION-DEHYDRATION METHOD FOR CRYOPRESERVATION OF CITRUS SHOOT TIPS

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Citrus is an economically important crop and in recent years one of the main goals has been to explore ways to improve methods for germplasm preservation. Among the currently available methods for long-term conservation of clonal germplasm like Citrus, cryopreservation of shoot tips is the most reliable, cost- and space-effective option. In this study, *in vitro* shoot tips of two Citrus clones were successfully cryopreserved using droplet-vitrification and encapsulation-dehydration methods. For both clones, droplet-vitrification induced significantly higher regeneration rates, reaching 53.5% for 'Frost Eureka Lemon' and 50.3% for 'Cook Eureka', compared with encapsulation-dehydration in which recovery was 25.3% for 'Frost Eureka Lemon' and 22.8% for 'Cook Eureka Lemon', respectively. The viability of cooled samples was tested by micrografting using following culture on WPM (1/4 ammonium nitrate) medium overnight. In this study, we have got much higher recovery using droplet-vitrification method with modified PVS2 and PVS3 than encapsulation-dehydration method. Additional researches to optimizing the both methods and should be confirmed using additional clones of Citrus.
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GLASS STABILITY AND ANNEALING BEHAVIOUR IN PLANT VITRIFICATION SOLUTIONS NO. 1 AND NO. 3.

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Cryopreservation of biological specimens is currently performed for wide varieties of organisms, cells and tissues. Their viability and biological characteristics maintenance over long storage periods at low temperatures relies on the vitrification of their cellular content. Avoidance of ice formation both during cooling and warming has taken most of the researchers' work and effort, as the achievement of ice-free vitrification was considered guarantee of indefinite avoidance of deleterious changes. However, recent findings focused on certain systems and extended storage periods, shed doubts on this paradigm. Some specimens lose viability under conditions not directly explained by ice formation or previous treatments. The evolution of vitrified specimens is suspected to cause this loss of viability. Although glassy water is still considered unable to produce the feared and lethal ice crystals, and the extremely high viscosity precludes large-scale changes, other residual movements could be determinant of the observed changes. Glassy state stability has also attracted workers' attention, suspecting that the relaxation phenomena underwent by some glasses might affect cells and their components, such as biological membranes or nucleic acids. The relaxation behavior of relatively complex systems such as vitrification solutions (not to speak of real cellular content) is not completely known. To understand the fate of these solutions below glass transition (TG), a storage and annealing study was performed employing the well-known plant cryopreservation solutions No.1 (PVS1) and No.3 (PVS3), chosen for their clearly different vitrification behavior. Storage under their corresponding TG showed no significant effect on their vitrification parameters, even for as long as 60 days. However, an annealing protocol in which solutions were cycled between temperatures over and under TG, resulted in the apparition of annealing areas whose evolution with the target temperature employed in cycles could be studied. PVS3 glass was concluded to present further stability than PVS1 glass.

CRYOPRESERVATION OF PLANT GENETIC RESOURCES USING PVS3 VITRIFICATION– ONE METHOD FOR SEVERAL SPECIES

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Ex-situ gene banks maintain, propagate and distribute plant genetic resources. Beside extensive seed and field collections, the long-term cryo-storage became an important backup approach for species that can only be maintained vegetatively. For the routine application of cryopreservation in a gene bank, it is desirable to use a simple and quick method which is useful for a plenty of plant species. Therefore, we have developed a simple PVS3 vitrification/droplet vitrification protocol which is applicable for six genera of the *Amaryllidaceae*, the *Solanaceae*, the *Lamiaceae* and the *Asteraceae* family. The protocol comprises the pre-culture of explants in MS-medium with 0.3 M sucrose overnight, followed by dehydration in solution A for 20 min and the vitrification of explants using PVS3 at room temperature for 2 hours. Afterwards, the explants are put either into fresh PVS3 solution or into PVS3 droplets on aluminium foils which are transferred into empty cryo-vials before the tubes are plunged into liquid nitrogen. The period of cold hardening of the *in vitro* donor plants and the explant size (0.8 – 3 mm) were adjusted depending on the genus. After rewarming at 40 °C and washing in 1.2 M sucrose solution for 20 min, successful plant regeneration could be achieved for *A. sativum* (74 %), *A. cepa var. aggregatum* (48.5 %), *Solanum tuberosum* (70.8 %), different mint species (81.3 %), *Orthosiphon* (30.8 %), *Salvia* (53.4 %) and *Artemisia* (65.1 %). In Allium, potato and mint, plant regeneration occurred without callus formation and showed spontaneous rooting, while in Orthosiphon, Salvia and Artemisia regeneration was associated with callus formation and a limited rooting. The developed protocol is equally suitable for *in vitro* donor material as well as for field material such as bulbils or cloves in Alliums and enables the efficient cryopreservation of several plant species.

EFFECT OF CRYOPRESERVATION ON GERMINATION RATE AND ACTIVITY OF ANTIOXIDATIVE ENZYME OF ONION (*Allium cepa L.*) SEEDS

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In this study, cryogenic storage using liquid nitrogen (LN) was applied to preservation of onion (*Allium cepa L.*) seeds which have poor longevity and lose viability rapidly under sub-tropical condition. The onion seeds of 10 accessions were cryopreserved in liquid (-196°C) or vapour (-180°C) phase of LN tank for 1 day with variable seed moisture contents (SMC) and germination rates (GR) using accelerated aging treatment for 5 days then examined GR. The initial moisture content (IMC) and germination rate (IGR) of seeds were regulated to range of 4~12 % and 65.3~98.7 %, respectively. Measurements showed that the GR of onion seeds after cryopreservation had no drastic decrease even in low initial viabilities except 3 accessions. In 3 accessions, the GR was decreased within 5% of IGR after seed cryopreservation of vapor phase. Almost seeds with 4~6% of IMC showed good storability as GR after cryopreservation of liquid phase and consequent thawing at the room temperature. Accelerated aging (AA) treatment (45°C, 95%

RH) was applied to seeds for control level of IGR before cryopreservation. Variable level of IGR showed no significant change after cryopreservation while AA treatment decreased seed viability in different degrees with varieties. The AsA/DHA and GSH/GSSG Ratios were not changed significantly after cryopreservation and the degree of change were different with varieties.
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FREEZING TOLERANCE OF PENNATE DIATOMS: POLAR VS. TEMPERATE STRAINS

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Polar environment is characterized by many extremes. Low temperatures, lack of liquid water, irregular nutrient and light supply, fluctuations in daily and annual cycles seem unfavourable for life. In spite of this, diatoms (*Bacillariophyceae*) are one of groups that adapted well and dominate in many polar habitats. Generally, microorganisms overcome unfriendly conditions via dormancy, but no morphologically different resting stages are known in freshwater diatoms. In this study, the tolerance of polar and temperate diatoms to freezing was experimentally tested and the difference in survivability of vegetative and resting cells was assessed. Diatom strains for the experiments were isolated in 2014 using natural samples from Maritime Antarctica (James Ross Island, Vega Island) and Arctic (Svalbard). Further strains were acquired from culture collections of microorganisms (CCCryo and BCCM). Resting cells were induced by incubation under nitrogen and light limitation in low temperature. The vegetative and resting cells of 26 strains were exposed to different freezing treatments to –4 °C, –20 °C, –40 °C and –180 °C. Treatments differed also in the rate of freezing and thawing (continuous versus abrupt). The study concludes that the diatom strains are sensitive to freezing. The freezing temperature had a significant effect on survival, but the results did not prove significant difference in survival between polar and temperate strains of diatoms, neither the importance of resting cells for the survival of freezing treatments. However, in the –20 °C treatment, polar strains showed higher viability in comparison with temperate ones suggesting that they are better adapted to the temperature that is habitual in their natural environment. Overall, the strains of *Pinnularia borealis* species complex revealed to be extremely resistant and survived even the liquid nitrogen treatment (–180 °C). This is the first study about diatom freezing tolerance comparing strains from polar and temperate habitats. Even though the conditions of the experiments were artificial, the study brought valuable data that could be useful for the introduction of diatom cryopreservation in culture collections. How do polar diatoms cope with their natural environment still remains an open question, which could be answered by a detailed field studies including multiple sampling throughout the year.