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SUMMARY REVIEW AND ABSTRACTS OF THE SLTB 2019 MEETING AND JOINT WORKSHOP ORGANISED BY THE STEM CELLS USER GROUP, ANDALUSIAN INITIATIVE FOR ADVANCED THERAPIES AND THE SOCIETY FOR LOW TEMPERATURE BIOLOGY

October 2nd - 4th, 2019, Universidad de Sevilla, Calle San Fernando, Sevilla, AL, 41004, Spain

SUMMARY REVIEW

The SLTB 2019 scientific meeting was held on 2-4th October, in the historic Antigua de Fabrica Tobacos building of the Universidad of Seville and was hosted by Professor Ramon Risco and Dr Ariadna Corral. The meeting included a workshop on cryopreservation of cell therapy products (2nd October 2019) run in collaboration with the UK's Stem Cell User Group and the Andalusian Initiative for Advanced Therapies. This represented an ongoing SLTB strategic objective to engage with other scientific groups interested in low temperature biology. Overall, the conference attracted nine invited keynote speakers and 34 free communications representing a scientifically dense programme dealing with diverse applications and cryobiological issues in cell preservation of a wide range of cells, tissues and organs.

CELL THERAPY WORKSHOP - jointly organised by the Stem Cell User Group, Andalusian Initiative for Advanced Therapies and the SLTB

The Stem Cells User Group, coordinated by Tamara Geach of the London Clinic, began with a valuable review by Mike Watts (Wolfson Cell Therapy Unit, University College London Hospitals, London, UK) on the importance of learning from errors made in the preservation of haematopoietic stem cells for medical treatments. Speakers, Karen Buckland (Great Ormond Street Institute of Child Health, University College London, UK) and Allison Hubel (University of Minnesota, USA) went on to explain the importance of careful scientific and technical approaches to the validation of new clinical protocols for the delivery of cryopreserved advanced cell therapies (page 159) . Kevin Jestice (Asymptote-General Electric Health Care, Addenbrookes Hospital, Cambridge, UK) continued in this vein to give examples of some of the challenges in assessing and validating new dry- and wet-thaw technologies and equipment for reliable recovery of cryopreserved products (page 160). Keiron Edwards-Pritchard (BioDock, Nottingham, UK) also described the key elements required in services to optimise appropriate cryostorage resources for cell therapies (page 160).

Subsequent speakers addressed the development of appropriate bionalytics in parallel with the application of new cryopreserved products (Stephanie Ingleton, Wolfson Cell Therapy Unit, University College London Hospitals, University College London, UK). The need for new technologies in monitoring long-term cryostorage (Lisa O'Connor, Cryobiology Stem Cell Facility, Trinity College, St James' Hospital, Crumlin, Eire) and Mick McLean (Atelerix Ltd, Cambridge, UK and University of Newcastle, UK) explored the real possibilities of novel technology for the shipment of cells for therapy stably in a non-frozen hydrogel.

The development of new cell-based advanced therapies were explored by Francisco Moniche (Hospital Universitario Virgen del

Rocio-IBiS, Vigo, Spain) for the use of bone marrow in the treatment of stroke and by Francisco Jose Calero (HU Virgen del Rocio – IBiS, Vigo, Spain) who described new developments in the utilisation of 3D bioprinting in cell therapy. New tools for the processing of cryopreserved advanced cell-based therapies such as the Miltenyi Biotec CliniMACS Prodigy automated cell preservation unit and the Zipthaw cryopreserved bag thawing device, were described respectively by Michael Schenk (Miltenji Biotec, Bisley, Surrey, UK) (page 162) and Terry McKindley (Deva Medical Electronic Ltd, Runcorn, UK); and Roberto Hanan (Cellulis, Santona, Spain) also described a novel mechanised DMSO-dilution/thawing device. Finally, in a review of regulatory developments in Europe, Inaki Iroke Oruezabal (Andalisian Network for Design and Translation of Advanced Therapies, Seville, Spain) illustrated the high level of activity in the development of clinical trials in cell-based Advanced Therapeutic Medicinal Products in Spain which in recent years had exceeded the numbers reported in Germany or UK (page 162).

SUMMARY OF KEY ISSUES DISCUSSED IN THE SLTB SCIENTIFIC MEETING

Gametes and embryos

Preservation of gamètes and embryos of non-human mammalian origin was addressed in a keynote, invited lecture by Christiani Ammorim who reviewed the state of the art in veterinary reproductive medicine (page 163). Cryobiology in human assisted reproductive technologies was addressed by a number of speakers, including the keynote lecture on the importance of the biological state of the mitotic spindle in successful preservation of human Embryos (Zuzana Holubcova, Masaryk University, Brno, Czech-Replublic) (page 163), genetic imprinting in embryos and oocytes (Miguel Gallardo (Universidad Hospitalaria Malo Clinic, Lisbon, Portugal) (page 164) and the effect of pre- and post-thaw incubation vitrified human oocytes on their maturation state and nuclear DNA structure (Irena. Kratochvilova, First Faculty of Medicine, Charles University, Prague, Czech Republic) (page 164).

Complex organs and tissues

Barry Fuller (Department of Surgery, University College London, UK) reviewed key historical events and developments in the preservation of human organs for transplantation (page 165) and noted in particular the key contributions made by the recently deceased Professor David Pegg to whom the 2019 SLTB conference had been dedicated by the local organiser Ramon Risco (Departamento de Fisica Aplicada III, Esculela Tecnica Superior de Ingenieria, Universidad Sevilla, Spain). In free communications on cryopreservation of more complex tissues the development of improved viability assays by confocal micrscopy for human venous allografts were reported by Pavel Mericka (Tissue Bank University Hospital, Hradec Kralov, Czech Republic) (page 165) developments in biobanking of human tissue engineered products were reviewed by Oleksandr Gryshkov and Birgit Glassmacher (Institute for Multi-phase Processes, Leibniz Universität Hannover, Hannover, Germany) (page 166). In addition, free communications by Maooz Awan and Claire Selden (both of the UCL Institute for Liver and Digestive Health, University College London Medical School, UK) discussed respectively, post cryopreservation apoptosis in human hepatocyte organoids (page 166) and their use in a cryopreserved bioartificial liver support device (page 167). Further data on large scale preservation of encapsulated liver-derived cells (HepG2) (Brookshaw et al., UCL Institute for Liver and Digestive Health, University College London Medical School, UK) and liver-derived organoids (Erro et al., Institute for Liver and Digestive Health, University College London Medical School, UK) were also presented in poster presentations (page 183).

Challenging new work just begun by Juan Hernandez-Medrano (Department of Obstetrics and Gynaecology, School of Medicine, Nottingham University, United Kingdom) described new data generated to try to begin to understand myometrial function and viability following controlled-rate cryopreservation of ovine uteri (page 168).

Another level of biological complexity impacting on successful hypothermic (or subnormothermic) storage and preservation of therapeutic cells was presented by Yuri Peterenko (Department of Biometerials and Biophysical Methods, Institute of Experimental Medicine of the Czech Academy of Sciences, Prague, Czech Republic) (page 168). Dr Petrenko showed data

on the subnormothermic storage and cryopreservation of mesenchymal stromal cells (MSCs) from different tissues. Dr Petrenko demonstrated the in vivo functional and phenotypic diversity of the MSCs derived from different tissues and the complex cell populations within individual tissue samples (NB. the term MSC has been widely debated and an alternative term "tissue specific stem/progenitor cells" appears to provide a more accurate terminology). Taking a novel perspective on the use of cryobiolgical approaches to deselect undesired cell types in tissue populations, Anatoliy M. Goltsev (Institute for Problems of Cryobiology and Cryomedicine, National Academy of Sciences of Ukraine, Kharkiv, Ukraine) described an approach using -80°C treatment to preferentially eliminate malignant Ehrlich carcinoma cells in tissue cell populations (page 169).

Preservation of plant germplasm

Resolving the Complex patterns of potential epigenetic and genetic change in post-thaw plant germplasm was reviewed in a keynote lecture by Elena Gonzalez de Benito (Technical University of Madrid, Madrid, Spain) (page 169). Technical advances in plant germplasm resources, enabled by the technique of microdrop vitrification was reported by Hannes Wilms (KU Leuven, Leuven, Netherlands) (page 170). Industrial application of cryopreservation of *Actinidia* spp. (Kiwi fruit) for food products was described by Jayanthi Nadarajan (The New Zealand Institute for Plant and Food Research Ltd, Palmerston North, New Zealand) (page 170) and Raquel Folgado (Botanical Division, The Huntingdon Library, Art Collections and Botanical Gardens, San Marino, USA) reviewed cryopreservation success at the century old Huntingdon Desert Garden in the preservation of horticultural dessert plant species (170).

Poster presentations on the preservation of plant materials also covered cryoconservation of hop pollen (Milos Faltus, Research Institute of Crop Production, Prague, Czech Republic) (page 182) , use of droplet-vitrification of shoot tips of the medicinal plant (*P. angulata* L.) (Romo-Paz et al., University of Guadalahra, Department of Botany and Zoology, CUCBA, Nextipac, Jal, Mexico) (page 182), cryopreservation of strawberry shoot tips for germplasm conservation (Balaraju et al., National Agrobiodiversity Center, Suwon, Korea) (page 181) and development of vitrification protocols to limit overgrowth by endophytic bacteria (Canizares et al., Department of Biotechnology-Plant Biology, ETSI Agronomica, Alimentaria y de Biosistemas, Universidad Politecnica de Madrid, Spain) (page 181).

Marine germplasm conservation

New work in the development of germplasm preservation and utility in aquaculture of marine species was reviewed by Pablo Heres (Marine Biological Resources Functional Preservation services, Estacion de Ciencias Marinas de Toralla, Universidade de Vigo, Vigo, Spain) (page 172) and for commercial supply for natural aquaculture of mussels by Estefania Paredes (Universidade de Vigo, Vigo, Spain) (page 171).

Cryoprotectant development

A new concept of the 3Rs (removal, reduction and replacement) for managing the toxicity issues in the use of DMSO was reviewed by Iryna Buriak (Institute for Problems in Cryobiology, Kharkiv, Ukraine) (page 172). The development of DMSO-free cryoprotectants were reported for human cell therapy (Miroslava Jandova, Tissue Bank, University Hospital Hradec Kralov, Czech-Republic) (page 173). Work was also presented on novel CPAs based on natural deep eutectic solvents (Anita R. Jesus, Faculdade de Ciencias e Technologia, Nova University, Nova, Portugal) (page 173) and fructose oligosaccharides (Peter Kilbride, General Electric Healthcare, Cambridge, UK) (page 174).

Other speakers also considered the use of cell culture pretreatments including the positive effects of insulin growth factor 1 induced maturation on success of oocyte preservation (Taisiya Yurchuk, Institute for Problems of Cryobiology and Cryomedicine, National Academy of Sciences of Ukraine, Kharkiv, Ukraine) (page 175) and crystalline cerium dioxide containing media (Olena Polivanova, IPC&C, National Academy of Science, Ukraine) to enhance preservation and recovery success..

Subnormothermic storage and shipment of cells

Emma Buick (Life Science Group Ltd, Bedfordshire, UK) described the application of a storage solution which had been used for a range of cell types (page 179). Storage of non-frozen bone-marrow mesenchymal stromal cells were also the subject of a video 'free-communication'

submission (a first video presentation at SLTB meetings) in which Fatima Aerts-Kaya (Hacettepe University Center for Stem Cell Research and Development, Ankara Turkey) showed data describing successful subnormothermic storage and recovery of this cell type for clinical use (page 184).

Developments in new technology for biopreservation

New technological developments in human cell cryopreservation for therapy were addressed for thawing of serum-based products (Farideh Z Bischoff, FreMon Scientific, San Diego, USA) (page 176). Ramon Risco (Dept. de Fisica Aplicada III, Esculela Tecnica Superior de Ingenieria, Universidad Sevilla, Spain) described work carried out with his collegaues Juan Vargas-Mancilla (Universidad Medica de Alta Especialidad, Institutto Mexicano del Seguro Social, Leon GTO, Mexico) on the development of a novel fusion protein of (AFPIII-Penetratin) which successfully delivered the AFPIII cryoprotectant moiety into cell lines, tissues and organs (page 176). In an extensive review of thermal analysis to understand behaviour of water content in plant tissue, Milos Faltus (Research Institute of Crop Production, Prague, Czech Republic) described the utility of different forms of differential scanning calorimetry (page 177).

Quality assurance and data management in biobanks

The potential improvements in sample identification and security of cryostorage of vitrified gametes and embryos using for monitoring of radio frequency identification (RFID) tagged storage vials in situ, were presented by Giles Palmer (The London Clinic Wales, Cardiff, UK) (page 177).

Peter Kilbride (General Electric Healthcare, Cambridge, UK) presented data produced in collaboration with the Anthony Nolan Cell Therapy Centre (Nottingham Trent University, Nottingham, UK) which showed that sample sections of umbilical cord blood containers used for quality control produced different cryoprotection profiles, yielding quality control data which may be inconsistent with the characteristics and overall viability of the main therapeutic cell preparations. The study had enabled the development of differential cooling of the QC and therapeutic segments of the UCB bags to give more reliable QC results which could avoid unnecessary discarding of cell therapy products (page 178).

Computational and mathematical modelling

Computational modelling and optimisation of cryopreservation protocols using the BioDynaMo algorithm were described by Roman Bauer (School of Computing, University of Newcastle, UK) (page 178). The use of Boyle van Hoff (BvH) equations to model osmotic regulation has been a regular topic of presentations at SLTB meetings over many years and Dominic Olver (University of Saskatchewan, Canada) described a meta-analysis of such data to revisit the application and utility of such modeling and propose a modification of the linear BvH model which incorporates hydrostatic pressure as a mechanism to accommodate the non linear osmotic responses of cells (page 179).

CONCLUSIONS AND FUTURE MEETINGS

These notes outline some of the core issues addressed at the SCUG-SLTB joint workshop on cell therapy cryopreservation and SLTB scientific meeting. Details of individual presentations are given in the following abstracts, many of which include unpublished findings. Following concluding remarks by Dr Ariadna Corral regarding this very successful meeting with delegates from 12 countries (UK, USA, Spain, Canada, New Zealand, Korea South, Germany, Belgium, Ukraine, Czech Republic, Portugal and Turkey), Dr Corral also announced the winners of the SLTB cryobiology photographic competition and that their images would be displayed on the SLTB website www.sltb.info/. Dr Alasdair Kay (University of York and SLTB treasurer) also announced the establishment of an annual award (starting 2020) dedicated to Professor David Pegg, which would support successful applicants to visit collaborators to develop their skills or new collaborations.

Christiani Amorim announced that the next SLTB scientific meeting would be held in October 2020 at the University of Brussels in Belgium (https://sltb2020.com) and invited all delegates to attend and present their latest scientific data. The Stem Cell Users Group also has an active programme of regular meetings (for information contact Tamara Geach at T.Geach@thelondonclinic.co.uk). During the meeting the SLTB committee also announced that

SLTB would be co-organising a cell cryopreservation session at the annual scientific meeting of the upcoming European Society for Animal Cell Technology-UK (http://www.esactuk.org.uk/index.php/meetings/2020/) as part of ongoing collaboration between SLTB and other societies and organisations interested in cryobiology.

Acknowledgements: The SLTB committee would like to thank the organisers Ariadna Corral, Ramon Risco and Tamara Geach. The organisers thank all speakers and session chairs, the UK SCUG community and the SLTB committee for their support in delivering another highly successful scientific conference.

ABSTRACTS

The Stem Cell User Group: an introduction

T Geach[1]

[1]The London Clinic, Stem Cell User Group (SCUG)

The Stem Cell User Group (SCUG) has been running since 2005 and was formed to respond to new legislation in the UK, namely the Human Tissue Act which arose in 2006 to regulate the bone marrow and stem cell transplantation sectors. It originally was geared toward helping laboratory and quality managers toward compliance and started as a small meeting comprised of centres from the Midlands and the South East of England. The format has always been to have two meetings per year in order for the community to have access to the most recent findings and opinions in the field. From the original 10 members there is now a large online e-mail based forum of 250 and there are still two meetings held per annum with a different host each meeting. The SCUG now has national exposure and has been held in centres throughout the UK and Ireland. This year is its first foray outside of the United Kingdom for The Society for Low Temperature Biology in Seville in October but it intends to continue in this vein with the June 2020 SCUG to be hosted in Cologne.

It is a not-for-profit organisation and seeks to be run at no cost to its members. Both the HTA and JACIE recognise that the meeting is the most applicable meeting for scientists, quality managers and all staff involved in stem cell and bone marrow transplantation for educational and CPD purposes. Recently the huge interest in cellular therapies has had an impact on the diverse nature of presentations this meeting has to offer. It is an evolving meeting and one that is growing in interest.

Failure of cryopreserved peripheral blood stem cells to ensure engraftment resolved by functional assays but not by post-thaw cell viability

M Watts[1], S Ings[1], C Balsa[1]

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The majority of patients receiving cryopreserved haematological stem cell support after myeloablative therapy recover an absolute neutrophil count (ANC) of 0.5x109/l within 14 days post-transplant (1, 2) and post-thaw viable CD34+ cell counts often used to test the product for cell potency (3). In an engraftment incident in 2013 however, the ANC recovery of eight children receiving stem cell support was > 30 days despite adequate viable CD34+ cell doses over 2.5x106/kg in all cases (4). No cells remained from the affected patients but five stored clinical stem cell products were tested and showed similar viable CD34+ cell recovery with a median (range) of 82%(65-95%). Colony Forming Unit-Granulocyte/Monocyte, (CFU-GM) colonies were additionally performed as a functional assay and failed to grow or were extremely poor in all samples. To test the freeze protocol used, individual stem cell harvests were prepared at clinical scale, divided in three, and cryopreserved in parallel in two laboratories. In one centre, the controlled rate freeze (CRF) protocol used for the patients and by 'passive' freezing1 at -800C in both centres. The freeze rate of the samples using the passive method was -10°C/min and CFU-GM recovery was equivalent between both laboratories with a median (range) of 70% (47-118%) for all samples. In contrast, the freeze rate of the CRF samples was -50°C/min and no CFU-GM growth was detected in four experiments. Retrospective patient review 2003-2014 showed a post-transplant median ANC recovery of 13 days but in 26/181 (14%) was > 30 days. The passive freeze method was adopted in 2015 and the median ANC of the next 35 patients

was 11.5 days, with none over 18 days (P<0.0001) confirming clinical potency with the slower freeze rate (4). The potential of sub-lethal freeze damage and 'viable' but non-functional cells has important implications for the QC of all cryopreserved cell therapies.

(1) Watts MJ et al. (2016) *Br J Haematol* **175,** 673-676. (2) Watts MJ & Linch DC (2016) *Br J Haematol* **175,** 771-783. (3) Yang H et al. (2005) *Bone Marrow Transplant* **35**, 881-887. (4) Morgenstern DA et al. (2016) *Br J Haematol* **174**, 942-951.

Cryostor CS5 stability validations

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Autologous gene modified CD34+ haematopoietic stem cells are used to correct monogenic disorders of the immune system. These advanced therapy medicinal products (ATMPs) are typically cryopreserved and stored for >1 month prior to infusion. We have developed a technique for effective formulation in a novel cryoprotectant Cryostor CS5® (CS5), which contains 5% DMSO and is manufactured by BlueLife solutions. CS5 has been accepted as a novel excipient for >10 clinical trials globally and to date >20 ATMPs formulated in CS5 have been infused in patients at Great Ormond Street Hospital (GOSH).

Currently we have two controlled rate freezer programs in use for CD34+ ATMP clinical trials and we have carried out similar validations for both programs. Cells are formulated in 20 mL +/- 1.5mL per bag at a concentration of 2-17.5 x 10^6 TNC/mL. Cells were filled into Kryosure 20F bags (Saint Gobain), overwrapped, cryopreserved and stored in Vapour Phase LN2. Stability validations have shown CD34+ ATMP formulated and stored in this way are stable, with similar values for cell number, viability, potency and colony forming units (CFUs) evaluated at 1, 3, 6 and 12 months post cryopreservation. Sterility tests were also negative at all time-points assayed.

The data presented supports the ongoing use of CS5 as a cryoprotectant for CD34+ ATMPs and we propose the implementation as a convenient excipient for other 'routine' bone marrow transplant (BMT) products.

Optimizing freezing profiles

A Hubel[1]

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Cell therapies are becoming a standard of care for the treatment of disease and injury. Unlike other standard therapies (i.e. drugs and medical devices), cell therapies have a complex supply chain that requires viable, functional cells all along it. It is noteworthy that what happens along the supply and processing chain determines the quality of the product at the end. Cryopreservation of a cell therapy is a common method of stabilization along this complex supply chain. Cryopreservation typically requires 6 steps: (1) pre-freeze processing, (2) formulation and introduction of a cryopreservation solution; (3) freezing; (4) storage; (5) thawing and (6) post thaw characterization. The focus of this work will be on optimizing the freezing process. It has been known for almost 50 years that the rate of cooling has a strong influence on the post thaw recovery of cells. Controlled rate freezing is often used to control the temperature as a function of time for cells being cooled; therefore improving both the recovery and consistency of outcome. A controlled cooling rate protocol has 5 steps: (1) initial equilibration; (2) cooling; (3) seeding of the sample; (4) secondary cooling and (5) cooling to the final temperature. The initial equilibration stage of the freezing protocol enables samples placed in the controlled rate freezer to equilibrate with the freezer. Optimizing this step, in particular, helps to improve reproducibility of the freezing process. The cooling rate for the sample is the cooling rate used for both primary and secondary cooling of the sample. One degree C/min is a cooling rate commonly used for several cell types. The temperature at which ice forms in the extracellular solution also plays an important role in the post thaw survival of cells. There are several methods of controlling the temperature at which ice forms in the extracellular solution: manual and automatic

seeding. Manual seeding requires the use of liquid nitrogen or a chilled instrument to induce nucleation and this approach is commonly used with cell types that require precise control of the nucleation temperature. Automatic seeding results from a dip in the sample temperature in the region where nucleation for the purpose of inducing nucleation. Ultimately, the sample is cooled to a final temperature at which time it is transferred to a low temperature storage unit. It is important to note that debugging your freezing protocol is straightforward; you can at any time stop the process, thaw the sample and determine viability. This approach allows us to determine if a particular segment of the controlled rate freezing protocol results in cell losses and a basis from which to change and optimize the protocol. Developing strategies for optimizing and debugging freezing protocols will be critical in the development and implementation of high efficiency cryopreservation protocols.

Dry or wet thawing; dare to compare

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The requirements by regulators for therapeutic laboratories to provide evidence of thawing have prompted a re-examination of thawing methodology. The world has moved on from simple water bath thawing. A number of new 'dry' and automated cell thawing devices have come onto the market. All will need GMP validation studies to back up claims of action by the manufacturer. These are clinical devices that can have very serious adverse effects on cells if incorrectly used. Patient safety is of primary concern. This presentation concentrates on a real world comparison of large volume cellular thawing using a water bath and a newer automated 'dry' system.

There is a wish list of specifications for an automated thawing device used in the treatment of patients. These specifications must conform to current regulations. Of specific interest is the Asymptote Via-Thaw machine which seems to cover all the requirements.

Biodock and maximising cryogenic storage capacity

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BioDock is an industry leader in the storage of biological materials. With over 17 years of specialist biobanking experience, BioDock has developed a range of services to help clients such as NHS trusts, research institutions, and pharmaceutical and biotech manufacturers to maximise their storage capacity. With resources such as a dedicated medical courier service, multiple storage sites and readily-available backup equipment, each service is bespoke depending on the needs of the client and their samples. In order to design each service, BioDock considers the 'STEEP' principles to ensure the services provided are of the highest quality.

(1) Campbell LD et al. (2018) *Biopreservation and Biobanking* **16**, 3–6. (2) FACT-JACIE. *Hematopoietic Cellular Therapy for Product Collection, Processing, and Administration* 7th Ed March 2018.

Influence of cooling rate on clonogenic cell recovery

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Haematopoeitic stem cells (HPC) are routinely cryopreserved from patients to provide haematological rescue of planned myeloablative therapies. The procedure is widely successful but there is no standard cryopreservation method and this can introduce risk. For example freeze rates of -5°C/min or more in controlled rate freeze (CRF) protocols compensated for the latent heat of fusion or simple ramp steps have resulted in the loss of Colony Forming Unit-Granulocyte/Monocyte, (CFU-GM) activity and engraftment failure: (1,2,3) prompting further exploration of the effect of freeze rate on CFU-GM survival. No CFU-GM colonies were recovered at a freeze rate of -10°C/min in 6 HPC samples tested confirming the fast freeze risk. The effect of slowing the freeze rate was then

studied. In four clinical scale HPC experiments, the starting product was divided into four 100 ml samples and placed in a -80°C mechanical freezer ['passive freezing' (4)]. The freeze rate was reduced by increasing the total freeze volume progressively by placing the first 100 ml sample in the freezer alone and stacking the remaining three on 100 ml cryoprotectant bags of 1 x 100 ml, 3 x100ml or 7 x100 ml. The increased volume slowed the sample freeze rate from -1.2°C/min to -0.9°C/min, 0.5°C/min and 0.3°C/min respectively. The expected mean ±1SD (range) recovery of CFU-GM of HPC clinical samples is 68 ± 27% (27-104%) at our centre (5) and was 68 ± 27% (27-104%) for all samples tested at all freeze rates with no pattern of GM-CFC loss at the lowest freeze rate. A recommended freeze rate of between -1°C/min to -3°C/min has been proposed for HPC for clinical use but freeze rates as low as -0.3°C/min were not detrimental to CFU-GM survival in this study.

(1) Abrams RA et al. (1980) *Lancet* **2**, 385-389. (2) Gorin NC et al. (1983) *Eur J Cancer Clin Oncol* **19**, 485-491. (3) Morgenstern DA et al. (2016) *Br J Haematol* **174,** 942-951. (4) Watts MJ et al. (2016) *Br J Haematol* **175,** 673-676. (5) Watts MJ & Linch DC (2016) *Br J Haematol* **175**, 771-783.

Changes in viability markers of haematopoietic stem cells following long-term cryopreservation

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Hematopoietic stem cell (HSC) products for autologous transplantation are cryopreserved and stored in vapour phase liquid nitrogen (-197ºC to -150ºC). There is no consensus on a universal expiry date. Forty-four peripheral blood stem cell (PBSC) products that were in long-term storage for between 1 and 22 years and fifty-eight autologous PBSC products in storage for less than 1 year were included. A post-thaw sample was taken directly from the clinical product bag for analysis. Percentage Viability (PV), viable CD34+ cell count (VCD34) and progenitor cultures (CFU-GM/BFU-E) were assessed. Analysis of these one hundred and two HSC products revealed a statistically significant decrease in PV, VCD34 and progenitors over time. Post-thaw VCD34 and progenitor culture results were not shown to be better indicators of neutrophil and platelet engraftment than the results at harvest. An approximate 2% reduction in VCD34 per year of storage was observed, allowing for prediction of the post-thaw VCD34 and could aid in the determination of an expiry date for each product.

(1) Fernyhough LJ et al. (2013) *Bone Marrow Transplant* **48,** 32-35. (2) Spurr EE et al. (2002) *Cryobiology* **44**, 210-217. (3) Vosganian GS et al. (2012) *Cytotherapy* **14,** 1228-1234. (4) Winter JM et al. (2014) *Cytotherapy* **16**, 965-975. (5) Watts MJ & Linch DC (2016) *Br J Haematol* **175**, 771-783.

Storage and transport of cells and tissues at room temperature

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Atelerix has developed a method of encapsulating human cells and tissues in an alginate hydrogel that makes them practical to use, adaptable and easy to store and transport – even at room temperature. Used as a method of cell storage and transport, it overcomes the acknowledged problems associated with cryo-shipping. Cells are encapsulated by *in situ* formation of the gel for shipping in plates, vials or beads, and can be rapidly released from the gel by the addition of a simple buffer.

This talk will present several case studies of using the encapsulation approach for hypothermic storage of cells used for fresh cell therapies and for cell models used as research tools for drug discovery and testing.

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3D bioprinting of skeletal muscle constructs with different bioink and the use of bioreactor for stimulating

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Tissue engineering technology has recently expanded into various areas due to increased demand for biocompatibility, that is, tissue repair and regeneration using natural biomaterials. Bioprinting has the potential to deposit various materials and cells in a 3D matrix by layer-by layer deposition and mimic biological organs or tissues. Tissue engineering is a promising approach to repair tendon and muscle when natural healing fails, however there is no consensus to reconstruct musculoskeletal tissue. Here, we report our experience in bioprinting and the different phases of this technique to reconstruct musculoskeletal tissue and its simulation in a bioreactor.

A scaffold was designed with a rhombus in its interior to provide a support to the cellularized hydrogel. Four hydrogels were made based on collagen and with a cell density of $40x10_6$ cells/mL. After 3 weeks of culture, the bioprinted scaffolds were fixed for their later staining with Hematoxylin-Eosin and with AMLs. Moreover, we stimulate our bioprinted scaffolds with mechanical and electrical stimulation increasing the stimulus by week for 3 weeks.

Sixteen scaffolds have been created by bioprinting and the good histological behaviour of the collagen and alginate bioink has been demonstrated. This bioink showed a cellular viability of 63%, reaching 90% of viability in 50% of the scaffolds manufactured with this bioink. The collagen bioink presented a viability of 24%; the collagen and matrigel bioink 40% of viability and MEC 50% of viability. The staining with AMLS has been visualized, therefore the initial differentiation to skeletal muscle has been demonstrated.

We tested 4 bioinks, we have demonstrated that collagen and alginate bioink showed more cellular viability than the other bioinks. The staining has been achieved by using antibody of specific muscle proteins.

Isolation of virus-specific T cells after cryogenic storage for treatment of high viremia in immunocompromised patients

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For over 30 years HSC transplantation has remained one of the most efficacious ways to treat Haematological abnormalities such as leukemia and lymphoma. Through this time we have learned many lessons along the way. One of the main issues with HSC transplantation is the absence of the Immune system, which increases the risk of virus inactivation. With the ongoing success of cell therapy, recent advances in a process known as Cytokine Capture System (CCS) allows the stimulation and subsequent selection of third party donor virus-specific T cells for transfusion into immunocompromised patients with high viremia. The treatment has shown great efficacy in reducing viral load. However; one bottleneck remains, the lack of time between finding a suitable donor and the manufacture of virus-specific T cells for infusion into patients with an immediate clinical need. To overcome this issue, we at Miltenyi Biotec are in Collaboration with Hammersmith Hospital, London, to ascertain whether virus specific T cells can be manufactured from frozen apheresis. With the aim of creating a cryogenically stored "off the shelf" product that can be used immediately, reducing the delay in treating immunocompromised patients with high viremia. The aim of the talk is to introduce the cytokine capture system, as well as introduce the experimental outline of the project.

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Cell therapies within the Andalusian Public Healthcare System

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The Andalusian Network for Design and Translation of Advanced Therapies (AND&TAT) was created in October 2008, promoted by the then Regional Ministries of Health and of Economy, Innovation and Science with the vision for Andalusia to aspire to play a relevant role in the field of advanced therapies, so that research, development and innovation in this field become the drivers for scientific progress, improvement of health and future social and economic development.

AND&TAT represents an innovative and singular technological maturation platform with the mission of promoting the development of novel cell and gene therapies and to incorporate them into the public healthcare system in Andalusia. Together with multiple stakeholders, AND&TAT has developed a Research & Development program around cell-based therapies. More than 900 patients have either participated in the 26 clinical trials we have promoted so far or have received, an ATMP manufactured, according to GMP, in our public network of laboratories. AND&TAT has provided its experience in activities such as advising researchers in regulatory aspects, setting up the manufacturing of ATMPs, designing and monitoring Clinical Trials, identifying industrial partners and generating new public-private partnerships, among others.

Cryopreservation is a small but essential process at certain points in the ATMP supply chain, since it allows maintenance of a low temperature at which the ATMPs' therapeutic properties can be preserved and their usually short self-life extended. In this regard, not only is cryopreservation a crucial step in the storage of ATMPs, but also a critical need for transport and distribution of starting materials/finished products between the different supply chain sites (procurement, manufacturing and administration site).

AND&TAT has been working on developing innovative solutions in recent years. Two national projects in collaboration with biotech companies have been funded within the Spanish "Retos-Colaboración" national programme. The first of these projects aims to test the use of the disruptive cryopreservation technology developed by Cellulis S.L., Limbo™, on ATMPs manufactured within the AND&TAT's GMP laboratories network. The second project's main objective is to evaluate a novel platelet lysate supplement developed within the Andalusian Public Health Care System (International patent application number: PCT/ES2018/070370) as a substitute of FBS in the cryopreservation media.

Cryopreservation of ovarian tissue: an emerging technology for female germline preservation of endangered species and autochthone breeds

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Many hundreds of exotic species and domestic animal breeds have been lost over the course of the last few decades. In order to avoid a similar fate to other animals threatened with extinction, it is crucial to develop and apply rescue strategies to ensure their survival for the future. One option as a safeguard measure is the cryopreservation of the ovarian reserve: the primordial follicles. Currently, there are three alternatives to cryopreserve this population of small follicles: (i) whole ovary, (ii) ovarian tissue fragments, or (iii) isolated follicles, using either conventional freezing or vitrification methods. After cryopreservation, the samples can be transplanted or cultured in vitro, aiming to produce fertilizable mature oocytes. In this lecture, we will review the current status of the cryopreservation of the ovarian reserve of domestic mammalian species and non-endangered wild animals as a model for threatened and endangered species and autochthone breeds, and discuss new insights into techniques that can be applied in the future.

Vitrification of human eggs for fertility preservation - hype or hope?

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Cryopreservation enables the long-term storage of reproductive cells. The advent of this technique revolutionized the field of assisted reproduction. It not only maximizes the success rate of *in vitro* fertilization (IVF), facilitates clinical treatment management and makes fertility preservation possible for cancer survivors. While freezing of sperm and preimplantation embryos has become a routine IVF procedure with excellent clinical outcomes, cryopreservation of human oocytes remains problematic. The rationale for disappointing results is the unique nature of the female gamete and lack of fundamental research. The major factor underlying the notorious propensity of human oocytes to cryoinjury is instability of meiotic spindle, the fidelity of which is critical for faithful post-fertilization development. We use the combination of polarized light microscopy and fluorescent confocal imaging to study the dynamics of acentrosomal meiotic spindle during the vitrification and thawing procedures in human oocytes donated for research. A better understanding of the behaviour of division machinery in vitrified eggs and the way the spindle reconstitutes upon warming will help to optimize the timing of critical IVF procedures. In a broader context, improving our theoretical knowledge of human oocyte cryobiology is pivotal for refinement of current cryopreservation protocols, development of novel approaches to female fertility preservation and down-to-earth consulting of women seeking egg freezing for social reasons.

Human oocytes are ready for ultra-fast vitrification after two minutes of exposure to cryoprotectant solutions

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The standard preparation protocol to ready the oocytes for vitrification takes from 8 to 15 minutes, most of which consists on a long exposure to a non-vitrifying solution (n-VS) to allow for osmotic equilibration.

A reduction in the duration of the protocol is desirable to improve the workflow in the IVF setting and reduce the time of exposure of the oocytes to suboptimal conditions: solutions with high molarity at a low temperature.

We developed in MatLab a simulation of the flux of water and solutes through the plasma membrane of the oocyte using the 2Parameter formalism. The conditions were reproduced *in vivo* using microvolumes of the solutions, and the volumetric excursions of the oocyte were recorded in an inverted microscope. Ultimately, oocytes were subjected to vitrification with a closed carrier after different preparation protocols.

We compared a standard equilibration protocol (EP) of 10 minutes of exposure to n-VS (7.5% EG, 7.5% Me2SO) followed by 1 minute of exposure to vitrification solution (VS; 15%EG, 15% Me2SO, 0.5 M sucrose) with a short dehydration protocol (DP) consisting of 1 minute of exposure to each solution.

The intracellular molarity of the oocyte at the end of both protocols was similar (5.40M in EP vs. 5.37M in DP); oocytes prepared for vitrification with DP showed a lower normalized water content (26.6% EP vs. 17.7% DP). Unfertilized oocytes survived vitrification in high rates after short dehydration protocols (30/30 DP) and 27/27 three pronuclear zygotes prepared for vitrification with DP survived the process and 24/27 resumed mitosis after 24 hours of culture. These results show that the critical intracellular solute concentration necessary for successful vitrification of human oocytes and zygotes at currently attainable cooling and warming rates can be achieved in just two minutes.

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Critical defects in cryopreserved cell nuclei: DNA structure changes

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Cryopreservation of cells is a fundamental task for a wide range of applications. First in this work, a detailed description of cryopreserved cell nuclei was connected with selected cryoprotectant effectiveness. We simultaneously evaluated changes in DNA, chromatin higher-order structure, and nuclear envelope of untreated or cryopreserved frozen cells. To determine the importance of subcellular changes and freezing mechanism on cell survival upon freezing/thawing, we treated the cells with different types of cryoprotectants: dimethyl sulfoxide, trehalose and ApAFP752 antifreeze protein. Beside nuclear envelope quality, we identified chromatin condensation as an important factor that strongly manages cryopreserved cell survival. Different cryoprotective compounds induced chromatin condensation to a different extent, corresponding to their effectiveness. We simultaneously evaluated DNA defects and the higher-order chromatin structure of frozen and thawed cells with and without cryoprotectant treatment. We found that in replicating (S phase) cells, DNA was preferentially damaged by replication fork collapse, potentially leading to DNA double strand breaks (DSBs), which represent an important source of both genome instability and defects in epigenome maintenance. Results of our work bring deeper understanding of the freezing processes, especially their effects on cell nuclei, and will contribute to the rational design of cryofunctional compounds and applications.

The pharmaco-physiology of surviving hypothermia in the organ transplant pathway

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The start of organ transplantation in the 1960's identified a crucial need to be able to preserve organs outside the body for several hours, and cooling to hypothermic temperatures became the method of choice. Cooling reduced ischaemic injury to some degree, but introduced other biochemical challenges for the cells which needed to be mitigated in order to be able to reliably transplant a functioning graft. The outcomes of rewarming the organs could produce additional injury by what became known as Ischaemia / reperfusion injury. Physiologists and cryobiologists worked to control these injury processes by providing special solutions via organ flushing to reach the internal cellular components – and the science of organ preservation was born. The current talk will review the historical development of organ preservation solutions, and the increasing understanding of I/R injury over the past decades. This will be put in the context of organ sharing logistics as they have developed into the sophisticated clinical services of the 2010's. The move towards dynamic hypothermic perfusion in the past decade will be highlighted in relation to the organ preservation solution perspective, and new formulations of organ preservation solutions will be described. Lastly, other novel approaches to cold non-freezing organ preservation solution will be highlighted, and what this may mean for further developments of organ preservation solutions.

Assessment of viability of long-term stored cryopreserved allogeneic venous grafts using vital fluorescence dyes and confocal microscopy

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Aim: To assess viability of cells of allogeneic cryopreserved veins after storage for several years. *Methods*: The study was performed in eight great saphenous vein grafts harvested in brain death donors and sent to the Tissue establishment EU TE Code CZ 000427 (TE) in the cooled (+4°C) organ preservation solution (Celsior ,Genzyme ,The Netherlands). Decontamination in antibiotics according to van Kats (1), and controlled rate freezing in the cryoprotective solution containing 10%DMSO were performed in the TE within 24 hours (2, 3). Storage in the vapour phase of liquid nitrogen for three (2 grafts) and/or five years (six grafts) followed. Grafts selected for the study did not meet the criterion of sterility, other criteria required for clinical application were met. After thawing and removal from the cryoprotective solution the grafts were sent to the Imaging Methods Core Facility in the cooled (+4°C) organ preservation solution (Custodiol CE, Dr. Franz Kohler Chemie, GmbH, FRG). Each graft was divided into three segments, in one of them viability was assessed immediately; the remaining segments were cultured in the CO_2 incubator for 24 and 48 hours in the Dulbeco medium. The tissue slices obtained by perpendicular and longitudinal cutting of vessels and stained with vital dyes according to Johnson and Rabinovitch (4) were examined by confocal microscopy. *Results* : Immediately after thawing the mean cell viability in slices obtained by perpendicular cuts (PC) was 81%, SD 6.5, median 87.5%, in slices obtained by longitudinal cuts (LC) the mean viability of 73.5%, SD 4.8, median 76.5% was observed. After 48 hours of culture the mean viability in PC declined to 60.6%, SD 6.8, median 66.5 %, in LC the mean viability of 74.8%, SD 5.9, median 79% was observed. *Conclusion*: The results showed good preservation of cell viability after long term storage and good stability of results after short term culture.

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Towards biobanking of tissue-engineered products

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The production of tissue-engineered products (TEPs) holds great promise for biomedical applications. Cryopreservation is a strategy of choice to achieve reliable long-term storage of TEPs and their off-the-shelf availability. The goal of this work was to develop an appropriate method for cryopreservation of cell-seeded electrospun scaffolds, mimicking the native tissue. Fibre mats were prepared by blend electrospinning of polycaprolactone and polylactide (100:50). The UV-sterilized scaffolds (thickness 100±10 μm, fibre diameter 0.5-1.0 μm, sample diameter 16 mm) were seeded with SaOS-2 cells (1×10^5 cells/scaffold). The following cryopreservation parameters were optimised: 1) pretreatment with sucrose (0.05 M, 0.10 M, 0.15 M), 2) type of cryoprotective agent (10% dimethyl sulfoxide (Me2SO) alone or in combination with 0.3 M sucrose) and 3) equilibration with CPA before freezing (10, 30 min). The cell-seeded scaffolds were frozen on day 3. After equilibration with CPA, its excess was removed and the samples were frozen in 12-well tissue culture plates using a controlled rate freezer (cooling rate of 1K/min down to -100°C). After storage, the scaffolds were thawed in a 37°C water bath for 1min and recultivated for 24 h. The viability of cells was monitored using Calcein AM/EthD-1 as well as Resazurin reduction assay before freezing and within 1 week after thawing. The results showed high viability of cells on scaffolds during culture (95%). The pretreatment with sucrose led to steady decline in cell viability with increasing concentration. Resazurin assay proved that the cells recovered well after thawing. Application of shorter equilibration time, pretreatment with 0.1M sucrose and addition of 0.3M sucrose to Me2SO resulted in the highest cell viability after thawing (70%). Taken together, this work provides next steps towards efficient cryopreservation and biobanking of TEPs.

Post cryopreservation delayed cell death in hepatic organoids is linked to cell-matrix interactions

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Effective cryopreservation of organoids requires an understanding of the cell-extracellular matrix (ECM) interactions during freezing. The Bioartifical Liver Machine (BAL) is a tissue engineered extracorporeal organ support. The BAL biomass consists of alginate encapsulated liver spheroids (AELS). Successful cryopreservation of the BAL biomass is essential to its success as a tissue-engineered product. The liver spheroids produce their own ECM and have upregulated hepatic function compared to monolayer.

Focal Adhesion Kinases (FAKs) are a type of non-receptor tyrosine kinase involved in cell-ECM interactions; in normal cells these interactions are vital for cell growth, function and survival.

Alginate encapsulated liver spheroids (AELS) were grown in a fluidised bed bioreactor for 12 days until spheroid formation was achieved and cryopreserved by slow cooling (0.3°C/min) using 12% DMSO. Warming (about 9°C/min) was followed by washing to remove the CPA. AELS were then returned to culture for 48 hours. The formation of FAKS before and after cryopreservation of AELS was assessed by immunofluorescence. The FAKs were stained with an anti-vinculin antibody, spheroids were co-stained for actin (phalloidin) and nuclei (Hoechst) and imaged using a confocal microscope. Viable cell number was determined by nuclei quantification and live/dead staining.

The number of FAKs fell during the first 24 hours post-thaw. The spheroids were no longer anchored to the matrix. This detachment was accompanied by a 30% decrease in the viable cell number at 24 hours (n=3). These FAKs re-established themselves after 48 hours, accompanied by recovery of viable cell number (n=3).

These results link post cryopreservation cell death to detachment, commonly known as anoikis. Scaling up of cryopreservation protocols from cell suspensions to organoids, and eventually whole organs must consider how the essential cell-ECM interactions will be preserved.

An "off the shelf" cryopreservable regenerative medicine cell therapy (cATMP) for treating patients with liver failure - UCL BioArtificial Liver Machine

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Over 1 million people die yearly from liver failure globally. 90% patients do not get a transplant with 25% death on waiting list for Acute Liver Failure patients. Given time, the liver can regenerate and repair itself. Currently there is no effective means to “buy” that time. We developed a cryopreservable BioArtificial Liver machine (BAL), to treat patients with severe liver failure. The BAL, a truly regenerative medicine, is a cell therapy that provides a cell biomass as organoids which delivers liver function temporarily to patients whilst their own liver repairs. It was successfully tested in large animal trials in porcine acute liver failure (ALF).

The logistics of delivery of any regenerative medicine or cell therapy are key to its commercial success. In terms of cell therapies, and particularly in those whose disease target has rapid and severe onset, the ability to cryopreserve the product at clinical scale is of utmost importance.

The in-vivo data we present illustrates the range of functional improvements demonstrated when testing the BAL, such that coagulation function was improved, intracranial pressure diminished, vasopressor requirement markedly reduced, and acidosis corrected.

Mechanisms that impact on cell recovery after cryopreservation have been explored, with respect to recovery of viable cell number. Presenting data on large scale cryopreservation of cell organoids, using >1 litre volumes, we have developed cryopreservation protocols which lead to good recovery of function, indicating the logistics would be suitable for GMP manufacture and clinical use. Successful cryopreservation has

been undertaken at volumes higher than any previously reported as far as we know.

In conclusion, using a regenerative medicine cell therapy approach to treat patients with acute, and acute decompensation of chronic liver failure, will reduce the need for organ transplantation in many patients, and enable better use of scarce donor organs.

Myometrial function and viability following controlled-rate cryopreservation of ovine uteri

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Uterine cryopreservation maintains viability and function, and represents a long-term storage option for transplantation (1, 2). However, it exhibited decreased myometrial contractile function compared to fresh controls. Here we investigate the effect of slow-freezing cryopreservation (SF) on uterine contractile function in response to increasing oxytocin (OT) concentrations.

Uterine-horns were perfused (0.5ml/min) with cold heparinised-solution followed by CPA (0.1 M sucrose, 1.5 M Me2SO, 10% FBS in Leibovitz-L15) for 60 (SF60, n=4) or 75 min (SF75, n=4) and cryopreserved using a controlled-rate freezer (Planer Ltd). Fresh uterine horns served as controls (CT; n=4). Non-caruncular tissue strips (0.5cm x 1cm) were dissected, mounted and kept in 25 ml-organ baths with KH buffer (37°C) and gassed with 95% O2 / 5% CO_2. Baseline tension and contraction frequency (peaks/min) and force (AUC) were recorded before and after OT cumulative doses (0-9.5 ng/ml)(3). Lactate dehydrogenase release (LDH; Roche) and connexin-43 (Cx43; Abcam) were used to evaluate tissue viability. Contractility data (LabChart v6) was analysed using ANOVA for repeated measures, while LDH and Cx43 compared using one-way ANOVA and Kruskall-Wallis tests, respectively.

Spontaneous ex vivo myometrial contractions were evident in CT but not in SF ($P<0.05$). OT increased frequency ($P<0.05$) and force ($P<0.05$) in CT with no response elicited in SFs. Conversely, OT increased basal tension in all tissues ($P<0.05$) but to a larger extent in CT ($P<0.05$). LDH release was higher in SF than CT ($P<0.05$), while Cx43 was lower in SF than CT ($P<0.1$).

In conclusion, cryopreservation decreases uterine function. This may result from increased tissue (LDH) and/or cellular damage (decrease Cx43) caused by cryopreservation or perfusion. New strategies to reduced damage and improve cryopreservation outcomes will be developed.

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Different aspects of clinical-grade processing of multipotent mesenchymal stromal cells: focusing the therapeutic properties depending on the cell source, hypothermic storage and cryopreservation conditions

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The opportunities to apply multipotent mesenchymal stromal cells (MSCs) in clinical practice have brought many challenges associated with the choice of most appropriate cell source, transportation methods and cryopreservation. Here we describe the thorough comparative analysis of therapeutic properties of MSCs derived from different sources, and provide some novel information on the clinical-grade hypothermic storage and cryopreservation approaches.

MSCs were isolated from human bone marrow (BM), adipose tissue (AT) and Wharton's jelly (WJ) in accordance with ethical guidelines and expanded in culture medium, supplemented with human platelet lysate. The immunophenotype of cells, their gene expression

and growth factor secretion were evaluated by flow cytometry, qPCR and multiplex assay, respectively. The immunomodulatory properties were assessed in co-culture with peripheral blood mononuclear cells (PBMC). The hypothermic storage of MSCs was performed at 4°C in buffered trehalose solution (BTS) (1). The non-toxic cryopreservation of MSCs was done using sugars pre-treatment approach (2) and cryoprotective solutions, containing sucrose, human plasma and DMSO. The therapeutic activity of cryopreserved cells was assessed on a full-thickness skin wound model in mice.

We found that the growth capacity was higher in WJ-MSC and AT-MSC cultures. On the other hand, BM-MSCs had the best capacity to suppress PBMC proliferation. Depending on the tissue source, the significant differences were found in the expression of PDL1, COX2, BDNF and HGF genes and in the protein level of HGF, NGF and VEGF within cell-derived secretome. The 72 hrs hypothermic storage of MSCs in BTS provided high MSC recovery rates and preservation of their differentiation and immunomodulatory properties. The cryopreservation of cells in human plasma, supplemented with sucrose and 1% DMSO resulted in 73% cell recovery. The cryopreserved MSCs using the cryoprotective medium as vehicle solution improved the healing of full-thickness wounds in mice.

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Freezing levels determine successful cryoablation of Ehrlich carcinoma cells

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During cryoablation in the center and on the periphery of tumor, a significant temperature gradient is observed. The tumor cells are exposed to different temperatures, which may cause incomplete tumor destruction. The research aim was to determine the intensity of necrotic/apoptotic processes and subpopulation composition of Ehrlich carcinoma (EC) after cooling down to different temperatures. The research object was EC cells, as a model of breast cancer, which were grown for 7 days in Balb/C mice peritoneal cavity. The EC cells were frozen in ascitic fluid at a rate of 10 deg / min down to -30°, -60°, -80° C, followed by warming in a water bath at 41°C. After thawing, the number of nucleated cells, their viability, and necrotic/apoptotic cells were determined using Annexin-V (BD, USA) and PI (Sigma, USA); subpopulation composition was examined using monoclonal antibodies (BD, USA) to CD44, CD24 structures. The positive controls were the indices of native EC cells, while the negative ones were those subjected to a direct immersion into liquid nitrogen.

Minimal cell viability (16.2 ± 0.7%) was observed when the sample reached -80°C, which was almost comparable to 90% cell death in negative control. In all the evaluated variants, the predominant way of cell death was necrosis, with the exception of freezing down to -30°C, whereat the number of cells in apoptosis increased significantly versus all the variants of freezing and controls.

The content of the CD44+CD24-, CD44+CD24+, CD44-CD24+ subpopulations significantly decreased with a reduction in end freezing temperature, while the least differentiated CD44hi were more resistant to low temperatures. The findings indicate among all the tested options, the cooling down to -80°C caused the maximum destruction of tumor cells.

Genetic and epigenetic stability after cryopreservation: is there real concern? Plant cases

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The development of efficient cryopreservation protocols is essential for cell, tissue or organ long-term storage. Efficiency of protocols should not only consider the recovery rates but also the genetic and epigenetic stability of the recovered cells. Cryopreservation procedures have been generally considered safe

and most reports account for the phenotypic and genetic stability of the recovered cells, tissues or organs. In plants, the origin of these variations is usually attributed to the application of *in vitro* culture techniques during the cryopreservation process rather than to the low temperatures used. However, more attention is recently given to the epigenetic status of DNA. Epigenetic changes modulate gene expression. Nevertheless, there are some evidences that changes occurred during the cryopreservation protocol could have long-term effects. Cases regarding microorganism, animal and plant cells, tissues, organs will be briefly reviewed.

Our group has been working for the last fifteen years on the effect of cryopreservation on genetic and epigenetic stability in plant species. Cryopreservation processes involve exposure to extreme physiological conditions, such as, not only low temperatures, but also low osmotic potential of the media and solutions used, the dehydration to which tissues are subjected to, or the use of potentially toxic and mutagenic substances such as DMSO. Results and conclusions from our studies on plant cryopreservation regarding how different factors can affect genetic and epigenetic stability in plants will be presented.

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Droplet vitrification: a success story for multiple crops, now including coconut

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The coconut tree (*Cocos nucifera*) is regarded as one of the most important palms in the world, with a yearly production of more than 59 M tones (1). However, while the production is increasing, its genetic diversity is under threat. The spread of diseases, pests and changing environmental factors endangers the genetic diversity not only in the natural population but also in the collections from the gene banks, since these are affected by the same factors. Coconut gene banks rely heavily on field banks since the coconut is recalcitrant. In the past years research was published on alternative methods of conservation such as cryopreservation of zygotic embryo's, which would offer a safe long-term solution. Yet, these techniques are still not widely used and not applicable to full grown shoots and with the prospect of preserving clonally propagated plants (e.g., by shoot culture or somatic embryogenesis) alternatives should be sought. This is why our research is focusing on the cryopreservation of shoot meristems. The droplet vitrification technique has already proven its value in other tropical crops such as banana and taro and was in this study applied to coconut (2; 3). Meristems were excised from in vitro shoots and exposed to PVS2 at 0°C ranging from 20 to 65 minutes and finally submerged into liquid nitrogen. After thawing, the meristems showed high survival rates around 86%. Subsequent regeneration was 0-20% with many of the shoot tips showing a "hollow" appearance after 2 months. Further experiments are thus needed to achieve higher regeneration rates.

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New Zealand's *Actinidia* sp. germplasm conservation using cryopreservation

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Kiwifruit is New Zealand's most important commercial fruit crop, with an export value of NZ$1.8 billion in 2018. Plant & Food Research holds one of the larger collections of kiwifruit germplasm outside China. With field germplasm constantly facing threats imposed by biotic and abiotic factors and it is impossible to replace some of New Zealand's unique genotypes, it is critical that we conserve these genetic resources. Ex situ conservation using seed storage and *in vitro* technologies including cryopreservation provide an alternative to field storage. Kiwifruit seeds have a high (c. 30%) oil content and differential scanning calorimetry thermal analysis

revealed that their lipids are in transitional phase (mixture of liquid and solid phase) at -20°C. Hence, we investigated if conventional seed banking at -20°C is optimum for storage of these seeds. For this, we compared seed germination and vigour following 12 months storage at 5, -20°C and cryopreservation for six species after drying to moisture content around 7% (fresh weight basis). We have also developed cryopreservation techniques for kiwifruit clonal germplasm conservation to complement seed conservation using droplet vitrification method. We have successfully applied this technique for shoot-tips of nine genotypes from five species, with regeneration ranging between 59% and 87%. Development of successful cryopreservation technologies ensures efficient conservation of New Zealand's kiwifruit germplasm for the long term.

An ultracool desert garden

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Desert plants, including cacti and succulents, are adapted to arid ecosystems worldwide where they live with minimal rainfall and extreme temperatures. However, a wide range of human activities are the main threats for many wild populations of desert plants, and their conservation in living collections is essential to maintain diversity. The Huntington Desert Garden, more than 100 years old, holds one of the most significant collections of cacti and succulent plants in the world. Nowadays, it displays more than two thousand species belonging to more than two dozen plant families. The Cactaceae, Agavaceae, and Aloaceae families are three of the most representative groups, and they all contain numerous endangered species.

A cryobiotechnology program was created at The Huntington to assure the long-term conservation of the Desert Garden collections. Therefore, we performed seed germination trials for cacti, agaves, and aloes to test their tolerance to liquid nitrogen. Most of the examined seeds showed tolerance to the liquid nitrogen exposure, and a seed bank is being created to maintain wild-collected accessions of threatened species. Although seed preservation is an excellent system to maintain the diversity of these plants, seeds of aloes and agaves are more challenging to obtain. Moreover, the field collections also contain clonal accessions with botanical and historical value. We developed cryopreservation protocols for several clones from different Agave and Aloe species to assure long-term conservation of relevant accessions, using droplet-vitrification based methods. Rooted plants regenerated from rewarmed meristems after liquid nitrogen exposure. Our work is leading to the creation of a germplasm bank that will preserve some relevant accessions from the Desert Garden collections.

From the ocean to the freezer and back

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The global production of blue mussel *(Mytilus galloprovincialis*) has a significant role on the marine aquaculture industry; hence it is one of the most cultivated mollusk worldwide (FAO, 2018). However, several hazards affect the culture and the seasonal supply of mussel seed, which sets up an increasing interest on the development of inland seed production without seasonal limitation and safe of global variations. Cryopreservation can provide the stable storage of living resource of mollusk early development stages throughout the year and the possibility of selection of genetic lines according to production issues. Research has normally focused on short term effects on post-thawed cells (1). Here, cryopreservation long term effects were analyzed on cryopreserved mussel larvae and their capacity for competent seed production using two different larval stages and protocols (2).

Mature blue mussels from Galicia (NW Spain) were spawned and cells were incubated to produce Trochophores (18-20h post-fertilization) and 72h-old D-larvae for these long-term experiments where larvae were incubated post-thaw in the lab on a full larval rearing until reaching the juvenile stage. Then those with higher survival rates were settled into culture ropes and moved into a mussel raft for a year-round monitored incubation in the ocean and the obtaining of a second generation (F2). The study of the cryopreservation long term effects is essential to achieve the totally implementation of

cryopreservation for Aquaculture and fish management.

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Cryopreservation of marine resources and its application on aquaculture industry and biodiversity conservation

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Many factors, such as environmental variations, the anthropologic activity, diseases, parasites and the past improper management threaten several marine invertebrate fisheries; some of them play an important role on the economy worldwide and/or have an important value in the ecosystem network. The present research is focused on the development of specific protocols and biobanking biodiversity as a tool for achieving the sustainability of invertebrate fisheries. Cryopreservation is suitable technique for marine conservation. The work carried out consisted of the development of successful cryopreservation protocols for sea urchin species (*Paracentrotus lividus*, *Echinus esculentus*, *Sphaerechinus granularis* and *Echinocardium cordatum*) and two clams of high commercial value *Venerupis corrugata* and *Ruditapes decussatus*. Cryopreservation will help overcome the seasonal limitations that affect the natural production and boost the implementation of biobanking for the aquaculture industry, by providing a reference back-up copy of a family for selective breeding or allowing crossing between different seasons (1). When tackling each species, toxicity tests were carried out to determine the suitable CPA and cryopreservation protocols were developed to study the larval tolerance to freezing. Further research is involved on the study of larval cryopreservation on other two marine mollusks which have high commercial interest: *Pecten maximus* and *Aequipecten opercularis*.

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The cryoprotectant DMSO. Identifying problems and developing its safe and effective use

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DMSO has long been the cryoprotectant of choice for cell and tissue cryopreservation. It is extensively used for cryopreservation of haemopoietic progenitor cells, bone marrow and others. DMSO has also been employed in medicine and veterinary applications as an anti-inflammatory drug. It is also used to enhance the penetration of therapeutic agents through cell membranes. However, despite its many benefits, DMSO toxicity and adverse reactions remain a topic of debate.

The aim of our study was to review the biological effects of DMSO and its toxicity with context to its effectiveness and value in the cryopreservation of transplants for cell therapy.

DMSO has a wide range of biological effects determined by its chemical nature. The effects of DMSO depend on its concentration, exposure time and cell type. DMSO is able to increase cell membrane fluidity. Membrane perturbations result in changes of cytosolic Ca2+ levels, function of Ca-dependent enzymes and translocation of specific messengers involved in cell differentiation. Impairment of mitochondrial membrane potential and inhibition of respiration have been also reported. DMSO can act as an apoptosis trigger. DMSO affects genomic processes in cells not only via specific messengers but also by changing DNA methylation profiles, up- or down-regulating different genes. DMSO has been reported to

cause adverse reactions in patients after transplantation of cryopreserved cells.

The methods of minimizing DMSO adverse effects and cellular toxicity are reduction of overall DMSO concentration, its removal or depletion before transplantation and replacement of DMSO with alternative cryoprotectants.

As with most drugs, DMSO can cause either positive or negative outcomes. To avoid possible adverse results of DMSO application as a cryoprotectant an understanding of its biological impact is required. These will be discussed as will further studies and the importance of the development of novel approaches to cryopreservation of cell grafts.

New cryopreservation technology of hMSCs – first preclinical results using DMSO containing or DMSO-free medium

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BACKGROUND: Development of Advanced Therapy Medicinal Products (ATMPs) based on human mesenchymal stromal/stem cells (hMSCs) has been a worldwide effort leading to new strategies in combating number of pathological conditions. This is reflected by increasing number of clinical trials evaluating cultured MSCs isolated from different sources. HMSCs have a tremendous potential for cellular therapy, regenerative medicine and tissue engineering, it is desirable to cryopreserve and bank them to increase their access and availability. OBJECTIVE: This research is a part of the clinical trial aimed on using ATMPs based on hMSCs in patients undergoing repeated total hip replacement. Cryopreservation technologies can help to solve situations when the patient is unable to undergo the operation, but cultivated hMSCs are harvested and prepared for application. The main goal of the research was to optimize the efficient cryopreservation protocol to achieve good specifications for cryopreserved hMSCs. We are also in the initial phase of the testing of new DMSO-free cryopreservation medium Biofreezee, Biochrom AG, Berlin, FRG. MATERIALS AND METHODS: To compare characteristics of fresh and frozen hMSCs we used trypan blue exclusion test (cell viability), flow cytometry (cell viability and phenotyping), sterility determining and the clonogenic assay of cell proliferation. RESULTS: Cryopreserved hMSCs showed good quality parameters after thawing in comparison with fresh hMSCs suspension. On using DMSO containing medium the viability was up to 90% in all cases. The cell purity determined by flow cytometry was also acceptable. All final products of the ATMP were sterile after thawing. CONCLUSION: We cryopreserved hMSCs to develop a new ATMP qualitatively matching the parameters of medicinal product obtained from native (fresh) cells. Our result show that the cryopreservation method described in this study is suitable for the ATMP production while allowing the cells to maintain their phenotypic features and viability at GMP-compliant values.

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New cryoprotectants based on Natural Deep Eutectic Solvents

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Deep Eutectic Solvents (DES) are a new class of solvents that have emerged in the last decade and can be described as a result of intermolecular hydrogen bonds between two or more compounds that at a specific and adequate molar ratio cause a strong depression in the melting point when compared to those of the individual components (1). When such

components are natural primary metabolites such as sugars, sugar alcohols, amines, and amino acids, DES are called Natural Deep Eutectic Solvents (NADES). These metabolites can be found in animals living in extreme cold environments and are involved in the hibernation process allowing them to survive during winter time (2, 3).

This work aimed at the use of NADES composed by these natural primary metabolites as cryoprotectants, as an alternative to DMSO.

The successful production of these eutectic systems was confirmed by polarized optical microscopy (POM), showing the existence of an amorphous material. Moreover, Nuclear Magnetic Resonance (NMR) data confirmed the strong interactions between the components of each NADES system through hydrogen bonds.

Furthermore, cytotoxicity was evaluated and IC50 of NADES determined in mammalian cells, with values ranging from 1.0-2.2 M. Differential scanning calorimetry (DSC) technique was also used to evaluate the thermal behaviour of these eutectic systems, showing the strong effect on the water crystallization/freezing and melting processes which proves that these NADES are able to reduce the formation of ice crystals. The work herein presented opens new possibilities in cryopreservation and broadens the spectra of cryoprotective agents suitable for cell storage under cryogenic temperatures, reducing the cell damage and improving cell survival after thawing.

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Fructose oligosaccharides as a novel cryoprotectant for mammalian cells

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Successful cryopreservation of mammalian somatic cells requires the use of cryoprotectants, among which dimethyl sulphoxide (Me_2SO) remains the most common. The cryoprotective abilities of Me2SO were first recognized 60 years ago (1). However, Me_2SO presents a cytotoxicity risk to cells, requiring precise and careful use: it must be added to cell suspensions under chilled conditions, washed out or diluted post-thaw. The long-standing demand to develop new cryoprotectants, which may be used instead of or in conjunction with lower doses of DMSO, remains unanswered.

Fructose-based oligosaccharides (FOS), demonstrating cryo- and lyo-protective abilities on bacterial cells (2), appear as a potential alternative to Me_2SO for the cryopreservation of mammalian cells. In this study, we have examined the use of FOS solutions as cryoprotectants in a range of mammalian somatic cell types. Cryoprotection by FOS was observed with Chinese Hamster Ovarian cells, Jurkat (immortalized T cell line), and CACO-2 (colorectal adherent) cells, with cell membrane integrity, Alamar blue functionality, and proliferation of the cells observed. Disparate cell types were specifically chosen to determine if FOS could be used more generally as a mammalian somatic cryoprotectant and was not cell specific. The optimal concentration and composition of FOS solutions were determined. To better understand their cryoprotective mechanisms, the relationship between the degree of cryoprotection and some physical properties of the FOS solutions (nucleation temperature, glass transition temperature, osmolarity, viscosity) was also investigated.

This work forms the basis of using FOS as a cryoprotectant for mammalian cells, and this group of sugars may prove a novel source for mammalian somatic cell cryopreservation.

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Fertilization rate and further embryo development of human vitrified mii oocytes matured in vitro from GV stage oocytes in medium supplemented with insulin Growth Factor-I

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Approximately 20% of oocytes obtained after controlled ovarian stimulation (COS) can be nuclear immature and be either at the MI or GV stages (1). Due to the lacking number of mature oocytes retrieved from the patients, immature oocytes can increase the total of available oocytes for current or future fertilization. In vitro maturation (IVM) and vitrification of the oocytes also could be used for fertility preservation for those cancer patients where hyper-stimulation cannot be applied (2). Thus, studying of in vitro matured vitrified human oocytes has a great value in infertility treatment by assisted reproductive technology. The aim of this study was to evaluate the maturation capacity and fertilization outcomes of *in vitro* matured human oocytes in medium supplemented with insulin growth factor-1 prior vitrification.

Immature GV stage denuded oocyte were collected after COS and underwent IVM in the presence or absence of IGF-I and cumulus cells, as well afterwards oocytes which reached the metaphase II stage were vitrified by Cryotop method. After warming the gametes were fertilized by ICSI and their fertilization rate and blastocyst formation rate were assessed.

Maturation, fertilization and blastocyst formation rates were (40.3% vs 87.2%), (23.5% vs 82.5%) and (0 % vs 58%) in oocytes cultured in the absence or presence of IGF-I and cumulus cells, respectively.

Our findings have shown that IGF-I can improve the oocyte maturation, fertilization rates of in vitro matured GV stage oocytes prior vitrification. Only in the case of the presence of IGF-I and cumulus cells the matured oocytes after vitrification and subsequent fertilization were able to form blastocysts.

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Fibroblast cryopreservation in nanocrystalline cerium dioxide containing media

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The actual cryobiological issue is cryopreservation protocol improvement to increase the quality of preserved material. A solution to the problem may be found in a toxic cryoprotectant (DMSO) (1) concentration decrease and use of effective protective components such as nanocrystalline cerium dioxide (NCD). It is known that NCD has explicit antioxidant properties, which are quite valuable for cryobiotechnologies (2, 3).

The mouse fibroblasts of L929 cell line were cryopreserved in the media containing 1% and 5% DMSO and NCD in concentration 1 g/l (experimental samples). The samples containing 1%, 5% and 10% DMSO were used as a control. Cell number in each sample was 1 million/ml, the sample volume was 1 ml. All samples were frozen with the rate 1 degree/min to down -40°C with further immersion into liquid nitrogen.

To assess the cryopreserved fibroblasts viability the fluorescent marker 7-aminoactinomycin D (7-AAD) was used. In control samples the number of 7-AAD positive cells was 73.02±1.20%, 52.11±2.03% and 15.09±3.40% respectively, in experimental samples it was 46.05±1.36% and 49.07±1.14% respectively. Thus, fibroblast cryopreservation with DMSO of low concentration is possible if NCD is used as a protectant component. The findings need to be comprehensively investigated in future.

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Efficacy of a novel precision dry thawing system: preservation of plasma anti/coagulation proteins necessary for use in the clinic

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BACKGROUND: Fresh frozen plasma (FFP) is an appropriate therapy for supplementing coagulation factors in bleeding requiring massive transfusion. Before transfusion, thawing of FFP is monitored closely to avoid contamination and uncontrolled or prolonged thawing (plasma protein denaturation). Thawing devices must avoid damage to the plasma bags and bacterial contamination while rapidly warming. In this study, we evaluated the effect of a novel precision dry thawing device (ZipThaw) on clinically relevant clotting factor activities. STUDY: The UCSD School of Medicine testing Lab received a total of 197 freshly collected (pre-freeze) or freshly thawed plasma samples prepared using a standard commercial thawing device and ZipThaw. The Lab conducted over 1,782 tests for the following factors: Prothrombin time (PT), International Normalized Ratios (INR), Activated-Partial-Thromboplastin time (aPTT) Factor VIII activity, Factor V activity, Protein C activity, Protein S antigen, Von Willebrand activity and Thrombin Antithrombin Complex (TAT) concentration. [Carried out on ACL TOP 700 Hemostasis Analyzer using manufacturer's reagents w/ validated precision CV of 5-10% within 4 hours of collection/thawing. Two-tailed, student t-test performed]. RESULTS: 100% concordance between the two thawing methods was observed, with no significant differences between the pre-freeze group and the two post thaw groups, slight increase in the mean TAT complex observed. Overall, the means of all three groups were within the normal range as defined by the assay. Further, the increased mean TAT is more notable with standard thaw method (33%, $p = 0.004$) then with the ZipThaw (18%, $p = 0.054$). CONCLUSIONS: We report for the first time non-inferiority between the ZipThaw dry thawing device and the leading commercial thawing device by preserving anti/coagulation proteins and minimizing activation of clotting cascade. The results support potential utility in plasma and cellular therapies requiring the controlled precision thawing.

Design of a biological cryoprotector based on antifreeze proteins linked to a protein transfection domain

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This work addresses the problem of the cryopreservation of cells, tissues and organs in a novel way, avoiding the formation of ice in the cytoplasmic and interstitial space of tissues and organs by means of the internalisation of the protein AFPIII-Penetratin.

We carried out the molecular strategy to build the recombinant protein based on AFP type III antifreeze proteins linked to a protein transfection domains named penetratin. Then we constructed, expressed and tested the effectiveness of the recombinant protein AFPIII-Penetratin, performed different experiments to determine the viability of the protein such as differential calorimetry, assessed its penetration capacity in cells, tissues and organs by immunocytochemistry and confocal microscopy, and determined the cryoprotectant capacity of AFPIII-Penetratin at different subzero temperatures in cell lines, tissues and organs.

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Thermal analysis methods as a tool for development of cryopreservation protocols

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Understanding the behaviour of the water content in plant tissues during cooling and heating is a key factor in developing a reliable cryopreservation protocol as high water content during these processes can result in sample injury (Zámečník, Faltus, 2011). Differential Scanning Calorimetry (DSC) thermal analysis methods such as standard DSC (DSC), Temperature Modulated DSC (TMDSC) and Quasi-isothermal Temperature Modulated DSC (QITMDSC) represent a viable tool that can improve the knowledge of the thermal events (Zámečník, Faltus, 2014) and so help to reduce ice crystallization and cell damage by frost and so contribute in the development of more efficient and reliable cryopreservation protocols (Volk, Walters, 2006). The standard DSC was used for routine measurement when dealing with samples with known thermal properties and non-overlapping thermal events. Amount of freezable water or glass transition were detected by the standard DSC after specific sample dehydration. The MDSC method was used for analysing samples with overlapping thermal events. The method helped to separate a total heat-flow signal into thermodynamic and kinetic events. The QITMDSC method was used for the exact measurement of heat capacity in equilibrated conditions that help to identify the sample state of matter. Here we intend to demonstrate how the use of these DSC thermal analysis methods can be applied in the development of cryopreservation protocols, what DSC methods are appropriate for samples with specific thermal characteristics, what thermal events can endanger sample cryopreservation stability, and what results can be expected using a specific thermal analysis method.

This study was supported by research projects of the Ministry of Agriculture of the Czech Republic QK1910277 and MZE RO0418.

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Improving sample identification and reducing risks during cryostorage of vitrified gametes and embryos using radio frequency identification (RFID) tags that operate while samples are immersed in liquid nitrogen

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Until recently, no radio frequency identification (RFID) label could operate reliably at low temperature suitable for prolonged gamete and embryo storage in liquid nitrogen. Here we describe a unique identification and audit system that can monitor the quantity and position of individual frozen samples stored in liquid nitrogen inside a Dewar. The system is in principal adaptable to any Dewar size and brand. While established methods of cryopreservation have been documented since the 1950s for semen (1) and 1984 for human embryos (2), in the last decade, vitrification has progressively become the method of choice for the cryopreservation of human oocytes and embryos owing to improved post thaw survival and pregnancy outcome (3).

Recent publications, however, predicting the effects of sub-optimum conditions for vitrified samples (4) during sample audits have raised concerns about operating procedures. In the UK it is mandatory for a 2nd person to verify all processes involving transfer of gametes or embryos and good practice dictates periodic audits which further involve removing samples from their immersed state (5).

Existing auditing methods are laborious, time consuming and serious shortcomings of interpreting standard labelling have been extensively addressed (6). RFID labelling and functionality in liquid nitrogen conditions would offer heightened security, safety and uninterrupted traceability.

We demonstrate here that in 56 test cycles, correct location of all frozen samples can be reported within a modified Dewar by a simple operation imitating a tank audit. Moreover, in a

further 96 blind studies a "misplaced sample" could be allocated instantaneously. Similarly. notification of the absence of a documented sample (missing sample) had been recorded in 100% of the trials (n=44).

We demonstrate the proof of concept in using specialized RFID tags in an industry finally coming to terms with a heightened awareness of risk assessment (7).

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Recovery and post-thaw assessment of human umbilical cord blood cryopreserved in quality control segments and bulk sample

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The cryopreservation of umbilical cord blood and cellular therapies often relies on cryopreserved quality control (QC) segments to assess the state of the bulk sample prior to its thaw. These QC segments are usually cryovials or sealed tubing which are cooled concurrently with the bulk sample, from the same starting material. These are thawed independently of the bulk sample to assess quality of the cryopreserved product. However, there is often variation between the QC segment and the bulk sample on thaw, which can lead to unnecessarily discarded therapies. In this study we have quantified these differences, finding a reduction in total viable cells of approximately 10% in the QC segments. Additionally, we have shown that CD45+ cells are more adversely impacted than CD34+ cells, which changes the population composition post-thaw. The QC segments were found to nucleate at a temperature 9.4°C lower than the bulk sample and this may be the cause of such variation.

In addition, we have explored the impact of different cooling protocols, including and excluding a 'plunge' step, and shown that these give equivalent results to each other, while maintaining the QC segment versus bulk sample differences.

Improving nucleation strategies between bulk samples and QC segments could improve outcomes and accuracy in not only cord blood based therapies, but more widely with other cell therapies.

Computational modelling and optimisation of cryopreservation protocols using BioDynaMo

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Cryopreservation of biological tissue is currently in its infancy. Approximately 2/3 of donated hearts and lungs cannot be transplanted, mainly due to lack of suitable preservation capabilities. Moreover, the synthetic growth of tissues for clinical purposes, as well as large-scale pharmaceutical testing would benefit from progress in cryopreservation. Cryobiologist Peter Mazur has pioneered the theoretical/mathematical modelling of cell freezing (1, 2). However, due to the complexity of the problem, the field still lacks a well-established computational component and so relies mainly on a trial-and-error approach.

The advances in computing technology have rendered it possible to incorporate highly detailed and large-scale models of biological tissues. Here, we present CryoDynaMo, an agent-based computational software for the modelling and simulation of slow-freezing of biological tissues. CryoDynaMo is an extension of BioDynaMo

(3,4), an open-source software platform for computer simulations of biological tissue dynamics. Along those lines, we demonstrate that CryoDynaMo can account for experimental findings using various cryopreservation protocols, in particular involving different cooling rates. Moreover, we present preliminary results of tissue viability, taking into account recent experimental results from murine neuroretina. Overall, CryoDynaMo constitutes a computational framework that allows to incorporate experimental data and optimise cryopreservation protocols.

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A Meta-analysis of the Boyle van't Hoff relation and new developments in osmotic regulation modeling

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The relationship between extracellular concentration and equilibrium cell volume is critical for mathematical modeling of cryopreservation protocols. The current paradigm is to use the linear Boyle van't Hoff relation (BvH) to predict cell size relative to external concentration. The BvH model assumes no regulatory volume decrease/decrease of the cell or hydrostatic pressure (turgor pressure) exerted by the cell membrane. However, it is well known that some cells exhibit regulatory volume control and others withstand significant transmembrane pressure gradients. Therefore, to verify if the BvH model is an appropriate for cells in general, we compiled 139 BvH data sets from 1964 until July 2019. We performed a Durbin Watson [DW] autocorrelation test and determined that the BvH model is highly autocorrelated (DW score 0.961 < 1.781crit[dL]). Additionally, we found that the BvH model may be appropriate in the hypertonic region (DW score 1.855 > 1.700crit[dU].). Because of this apparent insufficiency in the linear BvH model, we developed a new model that considers hydrostatic pressure (turgor pressure) as a mechanism to produce a nonlinear response. This model predicts the BvH relation to hold until some turgor point, after which the volume - inverse osmolality relation becomes non-linear according to the plane stress of the cell. This turgor model is found to work well as a general model, resulting in unautocorrelated best-fit residuals (DW score 2.02 > 1.790crit[dU]). We conclude that the BvH relation is inappropriate for cells unless experimentally validated and may only be appropriate for a specific osmotic region. Our model predicts cryoprotectant loading and unloading protocols are not optimized since they do not account for this effect.

Cell shipment without a cold chain

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For transportation, cells are typically shipped in a cryopreserved state, either using liquid nitrogen or dry ice. These methods present a range of logistic challenges, are expensive and often require the use of the cytotoxic cryoprotectant DMSO.

LSG Ltd is working with Coventry University to develop a novel cell shipment medium (CellShip), designed for transporting and storing cells at ambient temperatures. We have identified a defined, xeno free formulation that maintains cell viability over a period of 72 h, including transport by a commercial courier.

HEK293 cells were cultured using complete MEM at 37°C in a humidified 5% CO_2 atmosphere. For transportation, cells were dissociated and pelleted, the culture medium was discarded, and cells were washed once using CellShip. Cells were resuspended in CellShip at 1.4 – 2.3 x 10^6 cells per mL in 2 mL aliquots and shipped in cryovials using a commercial courier. Temperatures were monitored using temperature data loggers. Following the 72h transport/storage period, cells were counted and viability assessed, the cells were then recovered in complete MEM at 37°C in a humidified 5% CO_2 atmosphere. Cell numbers and viability were assessed at 24 h and 48 h.

Following the 72h shipment/storage period the mean fold-change in viable cell number was 1.03 (n=4), with a mean cell viability of 98.8%, suggesting that cell number and viability had been maintained. Temperatures recorded ranged

from 11C – 27.4°C, with the maximum range of 13°C in a single experiment. Following 24h recovery, cells showed a fold-change of 1.72, which increased to 2.84 by 48h, indicating that there was no lag in recovery, which is often seen following cryopreservation.

A method for transporting cells at ambient temperatures would simplify the supply chain and provide a cost-effective, xeno-free, non-toxic alternative to cryopreservation, benefitting researchers and the cell-based therapy market.

Vitrification of human oocytes: the relationship between incubation timing before freezing and after thawing with state of thawed oocytes and utilization effectivity

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Our work aims to determine the impact of incubation time before freezing and after thawing on utilization of vitrified human oocytes. We based our conclusions on a detailed post-thaw microscopic observation of the oocytes with different incubation time before freezing and after thawing and correlation of these results with a detailed analysis of the oocyte condition at the time of utilization. We showed that the oocytes vitrified after 7 h-long incubation post retrieving have significantly higher probability to be successfully utilized than the oocytes incubated for a shorter period. This could be explained by the observation that longer-incubated oocytes had already formed the meiotic spindle and separated chromosomes (i.e. reached the state typical for maturated oocytes in metaphase II) at the time of vitrification. Indeed, oocyte freezing before the meiotic spindle assembly only lead to poor utilization efficiency. The results thus demonstrate that freezing the oocytes too early may critically disturb the crucial process of spindle and chromosome post-thaw state.

Cells surviving the ice age

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In nature, a number of species can survive sub-zero temperatures while returning their functionality back to their physiological state at normal conditions. This property results from nontrivial and complex physical-chemical processes taking place in the organism and helping them to survive the ice age. In this key-note lecture I will discuss and give comprehensive information about the following topics related to tissue engineered constructs:

1. Is dimethyl sulfoxide safe enough for freezing of cells and cellular structures?
2. Is electroporation an efficient approach for delivery of sugars into the cells? How it can be applied to cryopreservation of tissue-engineered constructs?
3. Do the most commonly used controlled-rate freezers maintain the set cooling rates?
4. Is controlled ice nucleation superior for stem cells to survive the cryopreservation process more efficiently?
5. What are the most relevant methods to cryopreserve tissue-engineered constructs?

To facilitate cells and cellular constructs to survive low temperature storage it is important to consider a number of processes, which can only be understood combining the knowledge of different fields of science such as engineering, physics, biology, mathematics, and chemistry. Moreover, we do not forget achievements of our passed away pioneers in the field of cryobiology: Peter Mazur, David Pegg and Igor Katkov. With the ideas and achievements of the founding fathers of the science of modern cryobiology we are approaching a new era and thus are not afraid to tackle new and still existing problems in cryobiology.

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POSTER PRESENTATIONS

Development of cryopreservation procedures based on vitrification in Mentha sp. to minimize the growth of endophytic bacteria

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The conservation of germplasm of cultivated species with vegetative multiplication can be carried out through field collections, in vitro culture (with limited growth) and by cryopreservation. Cryopreservation allows the conservation of plant germplasm in the long term. Successful cryopreservation procedures for mint apices have been obtained by vitrification either using PVS2 or PVS3 vitrification solutions (1). However, one important setback in some mint genotypes is the presence of endophytic bacteria. Their presence does not disturb *in vitro* growth of shoots, but reduces the success of cryopreservation protocols. We have studied the use of the antibiotic cephotaxime in different steps of the cryopreservation protocol for apices of *Mentha aquatica*. The cryopreservation protocol applied was based on the droplet-vitrification procedure, and the results obtained with PVS2 and PVS3 were compared. The antibiotic was included in the media used during cold acclimation or in the recovery media. The use of cephotaxime in the medium used during cold acclimation combined with PVS3 resulted in higher recovery. The use of the antibiotic in the recovery medium decreased the presence of bacterial infection but did not have a significant effect on recovery when the vitrification solution was PVS3.

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Cryopreservation of in vitro grown shoot tips of strawberry (*Fragaria* × *ananassa* Duch.) genetic resources by droplet-vitrification

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This study describes an efficient and widely applicable droplet-vitrification following cryopreservation for shoot tips of strawberry (*Fragaria* × *ananassa* Duch.) cvs. ‘Wonkyo3114’ and ‘Gurumi40’. The shoot tips were precultured in Murashige and Skoog (MS) liquid medium supplemented with sucrose (0.3-0.5 M). Precultured explants were osmoprotected with loading solution (LS, C4) containing glycerol 20% and sucrose 20% for 40 min and exposed to dehydration solution (B5) containing 40% of glycerol and 40% of sucrose for 40 min at 25°C, and then transferred onto droplets containing 2.5 µl PVS3 on sterilized aluminium foils (4 cm × 0.5 cm) prior to direct immersion in liquid nitrogen (LN) for 1 h. The highest regrowth rate (%) was obtained when shoot tips were precultured with MS + 0.3M sucrose for 40 h at 25°C in both the cultivars. The viability of cooled samples, followed by culturing on NH4NO3-free MS medium supplemented with 3% sucrose, 1.0 g/L casein, 1.0 mg/L GA3, and 0.5 mg/L BA for 5 weeks and then cultured onto MS medium supplemented with 0.5 mg/L GA_3 for 8 weeks obtained more than 55% regrowth rate in cryopreserved shoots tips. This result shows droplet-vitrification would be a promising method for cryostorage of strawberry germplasm.

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Droplet-vitrification method for shoot tips of *Physalis angulata L.*

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Physalis angulata is a species of the Solanaceae family, of edible and medicinal fruit. Its phytochemical constituents (physalins and withanolides) have antiparasitic, anti-inflammatory, antimicrobial, and antitumor activity. Indeed, this validates their traditional medicinal use and demonstrates its tremendous potential for further development within the pharmaceutical industry (1). The natural distribution of the species has been affected by different factors, such as modern agriculture, and the lack of adequate protocols reduces the ex-situ conservation opportunities (2). Cryopreservation is an ex-situ conservation tool that ensures the maintenance and preservation of the original population variability (3). The present study focused on optimizing the droplet-vitrification technique for apical buds of *P. angulata*, from *in vitro* plants. Thus, apical shoot tips of 1 mm size were excised from 4-week-old micropropagated plantlets. Dissected meristems were exposed to loading solution for 20 min at room temperature, dehydrated with PVS2 for different times (0-75 min) at 0°C, transferred to aluminium foil strips and directly plunged into liquid nitrogen. For re-warming, aluminium strips were rinsed in unloading solution for 20 min at room temperature. Explants were transferred to recovery medium and kept in dark for one week and two more weeks under low light conditions (5 µmol·m-2·s-1). Optimal results were obtained after 30 min of exposure to PVS2, whereas shorted or longer dehydration times resulted in lower viability. Shoot tips regrew within one week, and new shoots regenerated within five weeks. Plant recovery was recorded two months after the re-warming, resulting in 90% of re-warmed meristems regenerated into a whole plant. This preliminary study showed that the droplet-vitrification method is proper for *P. angulata*. Further investigations will be performed in other Physalis species to assure the long-term preservation of these valuable plants.

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Cryoconservation of hop pollen (*Humulus lupulus L.*)

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The hop plant, *Humulus lupulus, L.*, is a dioecious perennial species, and only female clones are used for beer brewing. Cryopreservation of pollen is an additional way to conserve many species. In particular, it can be used as an alternative strategy to preserve the genetic variation and minimize problems of asynchronous flowering among accessions preserved in a germplasm bank.

Storage of pollen not only effectively overcomes the obstacles in crossing of parents flowering at different times and in different geographical locations, but also is an effective means to preserve the genetic diversity of plants. Multi-branched panicles of selected individuals of male hop clones (12/00, 12/06, 19/07) were collected in July 2019. Hop pollen grains were spontaneously released from panicles and collected into plastic test tubes in laboratory conditions. Thermal analysis of hop pollen was performed using differential scanning calorimeter (DSC) Q2000 (TA Instruments, USA) with refrigerated cooling system (RCS) in the temperature range from -90 to +20°C. The cooling rate was 10°C /min. Temperature-modulated DSC method was used during warming cycle with warming rate of 1°C/min. Temperature modulation was performed at 1°C amplitude of modulation and 60 second period. Aluminium, hermetically sealed pans were used,

sample size ranged from 5 to 20 mg and the purge gas was nitrogen. No significant endothermic or exothermic events were detected in all genotypes tested. On the other hand, the glass transitions were detected in all samples during warming cycles and they ranged from -35°C to -10°C. The half-height of sample glass temperature was -18°C, -20°C and -28°C in clones 19/07, 12/06 and 12/00, respectively. The determined thermal characteristics of all hop clones tested make their cryopreservation possible.

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Large scale freezing and thawing of 1Litre of alginate encapsulated HepG2 cell biomass using a cryobag: a viable proposition for use with the UCL Bioartifical Liver (BAL)

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For rapid delivery of the UCLBAL to patients, robust cryopreservation of large volumes (>1 L) alginate encapsulated liver spheroids (AELS) and optimal cryopreservation protocols are required. Controlled rate freezing provides an average cooling rate of -0.35°C/min for small volume samples (max ~100 ml), however larger volumes are not routinely used. Multiple large scale samples could be cooled simultaneously however, the effect on the cooling rates may differ spatially due to LN_2 vapor distribution. Warming of the biomass also requires optimization. Both processes significantly affect the post-cryopreservation viability and performance of AELS.

Cryobags filled with 20% glycerol were temperature monitored with thermocouples. 20% glycerol was used as a mimic of hydrogel beads, with the same thermal histories. Filled cryobags were cooled in a CRF (Kyro750-Planer plc). AELS recovery was assessed in cryopreserving cell beads in the centre of a cryobag filled with empty alginate beads making 1 L volume. Warming consisted of warming cryobag from -150°C to -80°C followed by submersion and agitation in a 37°C waterbath. AELS were re-cultured to recover. Cell number and metabolic viability were assessed by nuclei counts and fluorescent staining of vital dyes (PI & FDA).

Cooling rates for single cryobag samples on each rack tier were; top:-0.3483°C/min, middle:-0.3566°C/min & bottom:-0.3495°C/min, n=5. For 3 cryobags cooled simultaneously; top:-0.3529°C/min, middle:-0.3496°C/min & bottom:-0.3403°C/min, n=5. No significant difference was observed between either rack positions or individual/multiple cryobags simultaneously. Thawing of cryobags was completed consistently within 10 min. Recovery of AELS successfully restored pre-freeze viability and functionality.

Cryopreservation of 3 x 1 Litre cryobags was achievable, providing an efficient and cost effective process. AELS placed centrally within the cryobag were successfully recovered; large scale tests with full volumes are underway.

Does DMSO elicit toxicity in cryopreservation of liver cell derived organoid cultures?

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Liver disease kills >12000 people p.a. in UK; deterioration from liver failure is often rapid, so any solution must be readily available. A BioArtificial Liver machine (BAL), can provide liver function allowing a patient's liver to repair and regenerate. Cryopreservation of a BAL will enable fast delivery to patients. DMSO is widely used, with varying reports of cell toxicity (1, 2), but with little data on its effects on cell-organoids. Since thawing of large volume cryopreserved organoid biomass is lengthy, and leads to exposure of AELs to max 20°C, we explored

DMSO toxicity in organoid culture mimicking a typical thawing protocol.

Alginate encapsulated cell spheroids (AELS) cells at 13.2+/- 0.14 x10^6cells/ml beads, viability 96.9%, were exposed to 12% DMSO, 38% Viaspan for 10 min at 0°C, 20°C or 37°C. Cell viability was assessed immediately after DMSO exposure; viable cell number on re-culture for 24 h with 40% DMSO as a positive control.

There was no detrimental effect of 12% DMSO at 0°C, 20°C or 37°C for 10 min after immediate exposure; viability was 97, 97 and 96% respectively; after 40% DMSO, viability was 53.46%. Viable cell numbers at 24 h were: at 0°C 14.1x10^6cells/ml beads, n=3; at 20°C, 13.9, n=3; at 37C 14.0+/-1.8x10^6cells/ml beads, mean+/-SD, with viability >97%. In contrast 40% DMSO exposure resulted in loss of viable cell numbers to 0.378x10^6cells/ml, viability 7.5%.

Liver cell-derived cell organoids can be exposed to temperatures as high as 37°C without compromise to viable cell number. As expected DMSO at high concentration was immediately toxic. These data indicate that a protocol for organoid thawing in volumes of >1 Litre, can be achieved, suitable for a BAL biomass clinical protocol.

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Short-term hypothermic storage supports maintenance of transplantable hematopoietic stem cells

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Hematopoietic Stem Cells (HSC) are can be isolated from bone marrow, mobilized peripheral blood and umbilical cord blood (UCB) and give rise to all mature blood cells. Cryopreservation of HSCs include the use of dimethyl sulphoxide (DMSO) to protect cells during freezing, but may result in cellular toxicity during thawing and infusion in patients.

With the increase of clinical HSC gene therapy, the need for novel short-term culture and storage protocols has been increased. Short-term storage of HSCs under hypothermic conditions could also provide an alternative to DMSO-cryopreservation and facilitate transport of cellular products.

SUL-109 (Sulfateq) promotes storage of several cell lines under hypothermic conditions. Here, we cultured human CD34+ UCB cells and lineage depleted (Lin--) Balb/c BM cells up to 7 days in serum-free HSC expansion medium with hematopoietic growth factors. SUL-109 containing cultures were stored at 4°C for 3-14 days. UCB cells were tested for viability, cell cycle and reactive oxygen species (ROS). DMSO-cryopreserved Lin-- BM cells or Lin-- BM cells maintained for 14 days at 4^{o}C were transplanted into RAG2-/- Balb/c mice and engraftment was followed for 6 months.

Hypothermic storage of CD34+ UCB and lin-- BM cells in presence of SUL-109 resulted in a protective effect on viability, increased numbers of CD34+ UCB cells in G0/G1 phase, and decreased levels of ROS. Lin-- BM cells maintained for 14 days at 4^{o}C in presence of SUL-109 retained their long-term engraftment potential, although not to the same extent as DMSO-cryopreserved cells.

These data show that SUL-109, most likely through modulation of ROS levels, protects transplantable, long-term engraftment supporting HSCs during short-term storage under hypothermic conditions and may be used as an alternative to short-term HSC cryopreservation.