Evaluating the efficacy of selective inhibition of Arachidonate 15-lipoxygenase (ALOX15) during human semen cryopreservation in protecting freeze thaw induced sperm damage

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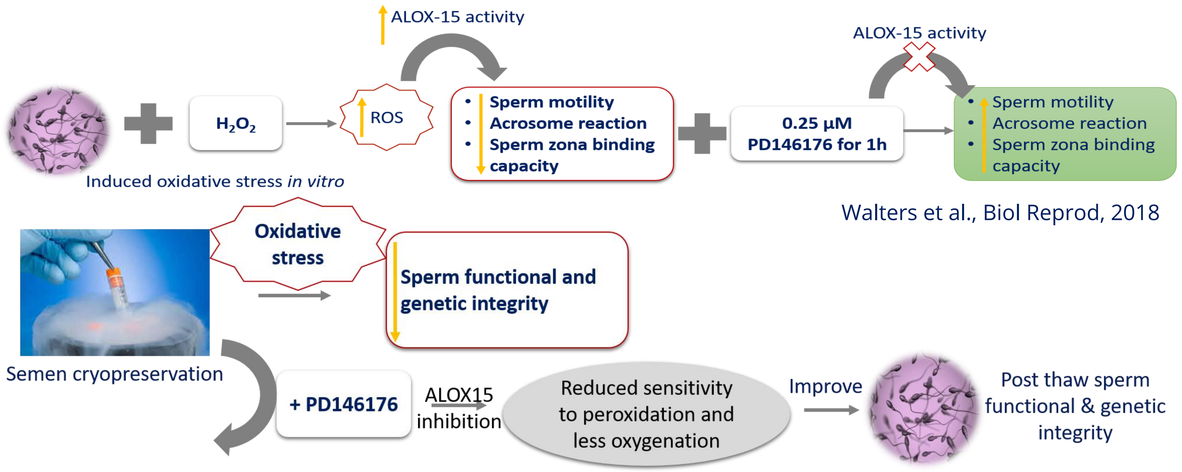
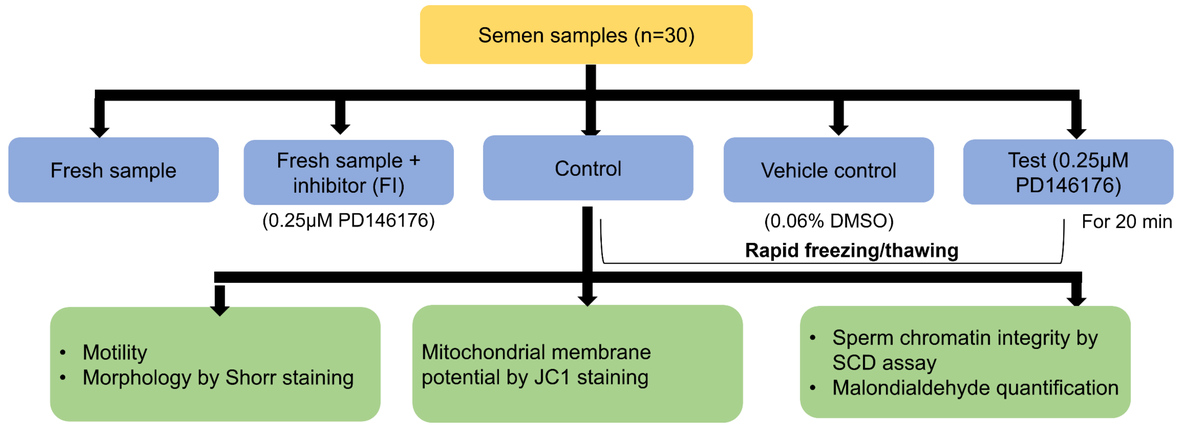
Background

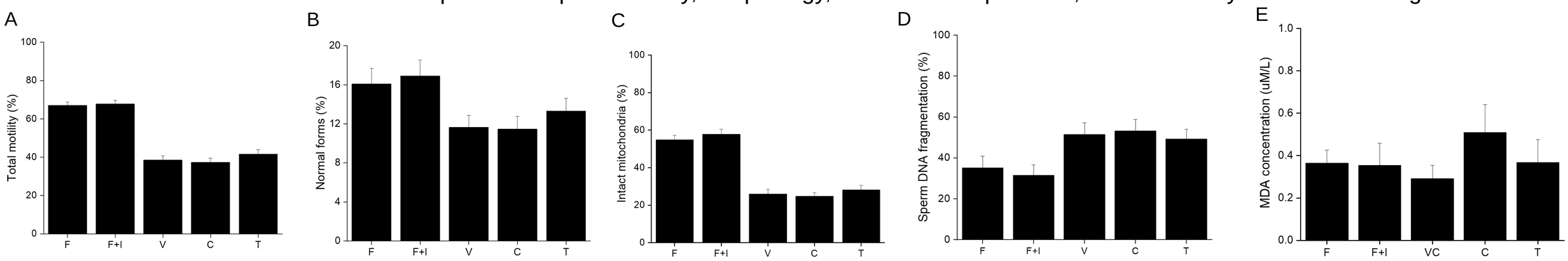


Methodology



Results

Effect of selective inhibition of ALOX-15 on post thaw sperm motility, morphology, mitochondrial potential, malondialdehyde level DNA fragmentation



Results demonstrated non significant improvement in A) sperm motility, B) number of sperm with normal morphology, C) number of sperm with intact mitochondria. In parallel, there was moderate reduction in the D) percentage of sperm with fragmented DNA and E) the level of lipid peroxidation product malondialdehyde in post thaw sperm when ejaculate was incubated with 0.25 µM PD146176 before freezing for 20 min compared to control.

Conclusion

Overall, the selective inhibition of ALOX15 during human sperm cryopreservation did not demonstrate any benefits in improving the sperm functional and genetic integrity

Acknowledgement

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POST-THAW QUALITY OF BEETAL GOAT SPERM CRYOPRESERVED DURING LATE SUMMER AND WINTER SEASONS

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Introduction

Cryopreservation is a complex process, as includes many critical stages and factors that may profoundly affect post-thaw sperm quality. Moreover, photoperiod is another factor that influences the reproductive activity of male. This study was carried out to determine seasonal variations in post-thaw quality of Beetal goat sperm.

Methods

Semen Collection

- Electroejaculation during winter and summer seasons
- Rams (n=03)
- Total ejaculates= 12
- 02 ejaculates per ram per season

Processing

- Pooled ejaculates were diluted with extender (Tris 300 mM, fructose 28 mM, citric acid 95 mM, 15% egg yolk and 5% glycerol).
- Final concentration = 200 × 10^6 sperm/mL
- Cooling to 4˚C over 2hrs
- Freezing and thawing at 35˚C

Attributes

- Motion kinetics
- Plasma membrane integrity (PMI)
- Viability

Results

Variables	Winter	Summer	p-value
Progressive Motility	8.4±0.5	13.0±0.7	0.005
Total motility	69.9±0.5	87.7±2.1	0.001
Viability	28.7±3.4	35.3±2.3	0.18
Total HOST +ve sperm	30.3±1.8	46.3±5.2	0.044
VCL	34.4±1.4	38.3±1.4	0.117
VSL	12.2±0.9	12.8±0.7	0.641
VAP	18.5±1.3	20.8±0.9	0.228
LIN	28.5±2.0	28.0±1.6	0.844
STR	53.8±1.4	51.8±1.6	0.418
WOB	49.8±2.1	51.3±1.5	0.605
ALH	1.8±0.0	2.0±0.1	0.018
BCF	4.0±0.3	4.1±0.2	0.766

QUALITY AND FERTILITY OF TREHALOSE SUPPLEMENTED CRYOPRESERVED STALLION SEMEN

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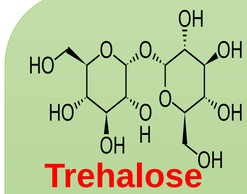
पशुधनं नित्यं सर्वलोकोपकारकम्

INTRODUCTION



Trehalose

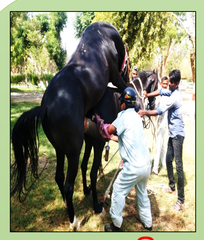
Nonpermeating disaccharide nonreducing sugar a) Non enzymatic scavenger b) Modulates membrane fluidity c) Osmotic effect (1,2)

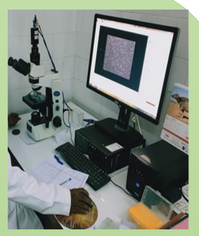
Enhanced cryoprotection of stallion sperms by Tre supplementation to semen extender.(3,4,5) Tremendous scope to increase the pregnancy rates for AI with equine frozen semen by improving freezability by adding Tre to Freezing Media.

OBJECTIVES

1. To study the effects of Tre supplementation to freezing extender on post-thaw seminal attributes, reactive oxygen species and lipid peroxidation levels of equine semen.
2. Comparison of pregnancy rates in mares inseminated by frozen-thawed semen cryopreserved with Tre.

MATERIAL & METHODS



Semen collection and evaluation

Subject 6 healthy Marwari horses 4 and 11 years.

Primary extender (Citrate-EDTA) for centrifugation.

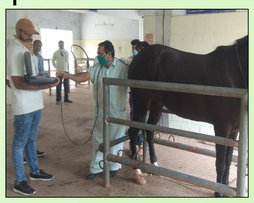
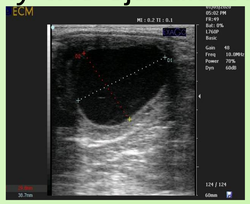
Secondary extender (Lactose-Glucose-EDTA-egg yolk) containing 5% DMF.

Semen freezing by vapour freeze technique.

Groups were Control SE w/o Tre, T1 SE with 50mM Tre and T2 SE with 150mM Tre.

Post-thaw evaluation Thawing at 37°C for 30 sec.CASA Progressive Motility(6), Viability(7), Membrane integrity(8), Acrosome integrity(9), Mitochondrial membrane potential(10), DNA integrity(11), Reactive oxygen species(12) and Lipid peroxidation levels(13) were estimated.

Fertility trial Semen doses were prepared with 50mM Tre concentration based on post-thawed parameters of forty-two ejaculates.

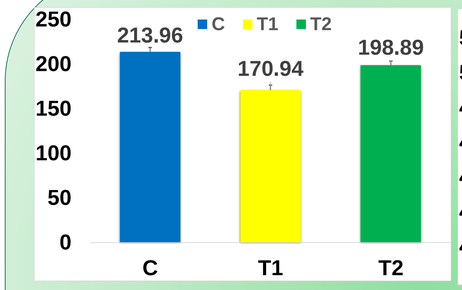
Mare management and insemination

Problematic mares were not selected. Examined by per vaginal examination, per rectal palpation and transrectal ultrasonography. Semen was deposited by mare AI catheter close to ovulation,15 mares were allotted in each group control and 50mM Tre. .

RESULTS

Post-thaw seminal attributes

Gr	PM (%)	SV (%)	MI (%)	AI (%)	MMP (%)	DI (%)
C	42.51^{a} ±0.98	48.36^{a} ±0.89	32.65^{b} ±0.43	72.87^{b} ±0.5	45.75^{a} ±0.93	87.21^{a} ±0.28
T_1	54.76^{b} ±1.11	60.03^{c} ±1.28	39.62^{d} ±0.61	76.89^{d} ±0.49	59.16^{d} ±1.11	90.25^{c} ±0.44
T_2	40.61^{a} ±0.98	48.01^{a} ±1	30.65^{a} ±0.45	70.16^{a} ±0.46	45.44^{a} ±0.82	87.09^{a} ±0.29



Post thaw ROS levels ((Hydrogen peroxide units per 2.5 million sperm) and LPO levels (nmol MDA/thousand million spermatozoa).

Conception rate

Pregnancy rates in control and Tre (50mM) groups were 46.66%(7/15) and 66.66%(10/15), respectively with overall mean pregnancy rates were 56.66% (17/30). NS difference between two groups.

CONCLUSION

Tre supplementation to freezing extender improves post-thaw quality of equine semen.

50 mM Tre is suitable concentration for cryopreservation of equine semen while using Lactose-Glucose-EDTA-egg yolk as SE.

ACKNOWLEDGEMENT

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LITERATURE CITED

1. Reddy, N.S.S., Mohanarao, G.J. and Atreja, S.K. (2010). Effects of adding taurine and trehalose to a tris-based egg yolk extender on buffalo (*Bubalus bubalis*) sperm quality following cryopreservation. *Anim. Reprod. Sci.* **119**:183-190
2. Bucak, M.N., Atessahin, A., Varisili, O., Yuce, A., Tekin, N. and Akcay, A. (2007). The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen: microscopic and oxidative stress parameters after the freeze–thawing process. *Theriogenology*. **67**:1060-1067..
3. El-Badry, D.A., El-Maaty, A.M.A. and El Sisy, G.A. (2017). The Effect of trehalose supplementation of INRA-82 extender on quality and fertility of cooled and frozen-thawed Stallion spermatozoa. *J. Equine Vet. Sci.* **48**:86–92.
4. Ghallab, A.M., Shahat, A.M., Fadi, A.M., Ayoub, M.M. and Moawad, A.R. (2017). Impact of supplementation of semen extender with antioxidants on the quality of chilled or cryopreserved Arabian stallion spermatozoa. *Cryobiology.* **79**:17-20.
5. Srinutiyakorn, D., Poonyachoti, S. and Tharasanit, T. (2017). The protective effects of trehalose on osmotic tolerance and quality of equine sperm during cold storage and cryopreservation. *J. Vet. Sci. Technol.* **8(7)**:30.
6.Ozturk C, Gungor S, Ataman MB and Ili P. Effects of arginine and trehalose on post-thawed bovine sperm quality. Acta Vet Hungarica 2017;65(3):429-439.
7. Pintado B, Fuente de La J and Roldan ERS. Permeability of boar and bull spermatozoa to the nucleic acid stains propidium iodide or Hoechst 33258, or to eosin accuracy in the assessment of cell viability. J Reprod Fertil 2000;118:145-152
8. Nie GJ and Wenzel JGW. Adaptation of the hypo-osmotic swelling test to assess functional integrity of stallion spermatozoal plasma membranes. Theriogenol 2001;55:1005-18
9. Watson PF. Use of giemsa stain to detect change in acrosome of frozen ram spermatozoa. Vet Rec 1975;97:12-15.
10. Glazar AI. Assessment of Sperm Mitochondrial Function: JC-1 and Rhodamine 123. Equine Reproductive Procedures, First Edition. Edited by John Descanio and Patrick McCue, Published by John Wiley and Sons 2014, p. 486-87.
11. Hughes CM, Lewis SE, McKelvey-Martin V and Thompson WA. Comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay. Mol Hum Reprod 1996;2:613-9
12. Hayashi I, Morishita Y, Imai K, Nakamura M, Nakachi K, Hayashi T, et al. High-throughput spectrophotometric assay of reactive oxygen species in serum. Mutat Res 2007;631:55–61.
13. Suleiman SA, Ali ME, Zaki ZMS, El-Malik EMA. and Nasr MA. Lipid peroxidation and human sperm motility: protective role of vitamin E. J Androl 1996;17(5):530-537.

CONTRIBUTION OF METABOLIC PATHWAYS IN RESISTANCE OF ALGINATE ENCAPSULATED MESENCHYMAL STROMAL CELLS TO STORAGE AT AMBIENT TEMPERATURE

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CRYO2021 VIRTUAL CONFERENCE | JULY 20-23

Introduction

Mesenchymal stromal cells (MSCs) implementation requires convenient and efficient storage methods:

Clinical center(s)
Tissue sample collection
Cell-based therapy

Research center(s)
Cell Isolation & Expansion
Researches

Approaches for storage & transportation of cell product

Cryopreservation
is widely accepted but has some disadvantages:
- expensive equipment;
- liquid nitrogen for transportation;
- toxic cryoprotective agents;
- washing-out procedure in clinic

Storage at ambient temperature
may solve problems associated with cryopreservation.
But some questions are still open:
- the period of effective storage;
- the most beneficial for storage state of MSCs (monolayer, suspension, alginate encapsulated);
- cell properties during storage

The objective was to study **viability** & **metabolic properties** of human dermal **MSCs** in **monolayer**, in **suspension**, and **encapsulated** in **alginate microspheres** (**AMS**) under **culture** conditions and during **storage at ambient temperature.**

Design of experiment:

The MSCs have been obtained in compliance with the rules of biomedical ethics after written informed consent of the donor in accordance with the Helsinki Declaration of the World Medical Association for Biomedical Research and with the requirements of the IPC&C Ethics Committee

Expanded **human dermal MSCs**
α-MEM (10% FBS), 5% $CO_2$95% humidity

Monolayer **Cell suspension** **AMS**

Culture

Storage^ at 22 °C in sealed containers in α-MEM with 10% FBS

Viability & metabolic properties assessment

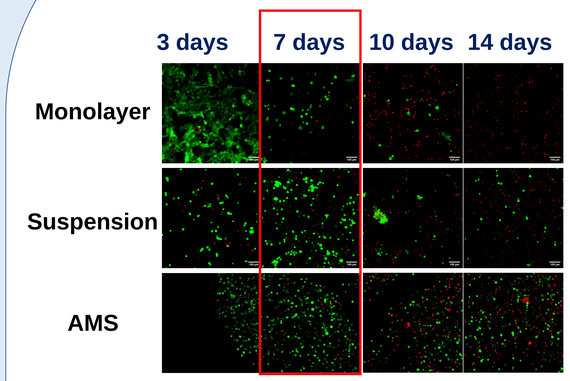
We have studied:
- **Viability by FDA/EB dual staining**
- **Metabolic activity by Alamar Blue test**
- **Actin filaments (Phalloidin-FITC)**
- **Adhesion ability**
- **Cell cycle**
- **Mitochondrial membrane potential (JC-1 test)**
- **ROS level & resistance to hydrogen peroxide**

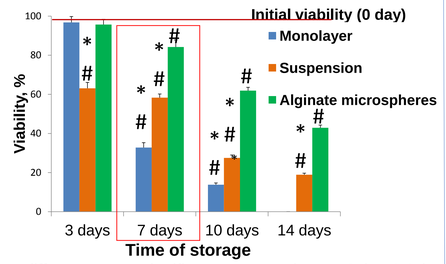
^ - Cells in monolayer and in AMS were precultured before storage

Results:

Storage at 22 °C

Viability of MSCs by FDA/EB staining



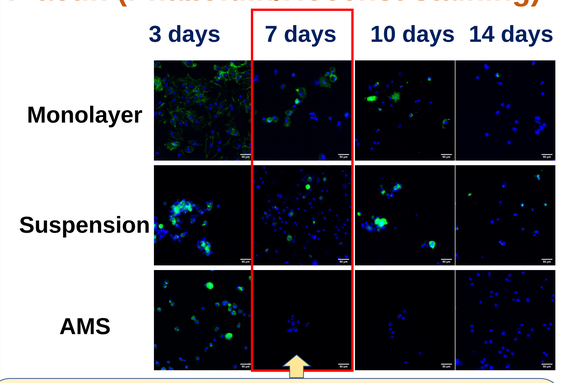


*- differences are significant compared to AMS ($p < 0,05$)

- differences are significant compared to initial index rate ($p < 0,05$)

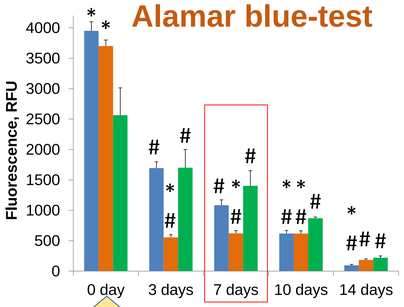
Alginate encapsulation significantly supported MSCs viability during storage, while it was decreased dramatically in both monolayer and suspension.

F-actin (Phalloidin/Hoechst staining)



F-actin was revealed in MSCs in monolayer & suspension even at 14 day of storage but it was not detected in encapsulated cells starting from the 7th day.

Metabolic activity by Alamar blue-test



Alginate encapsulation decreased basal metabolic activity in MSCs in preculture period. Metabolic activity decrease in AMS during subsequent storage was smooth compared to the sharp decrease in monolayer and suspension.

After 7 day of storage MSCs were returned to standard culture conditions. **Adhesion ability** was assessed after 24 h and given below:

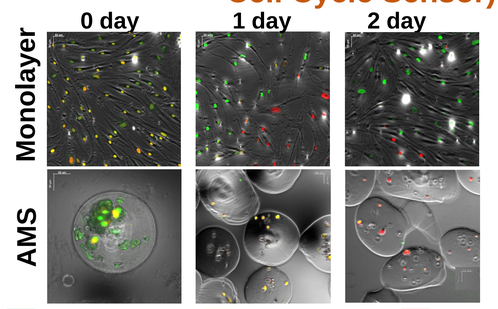
Suspension 12±0.8 %

AMS-extracted cells 64±3.6 %

After 7 days of storage cells extracted from AMS were able to attachment and proliferation in culture.

Culture

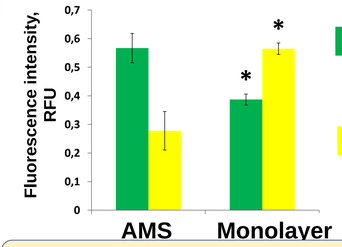
Cell cycle analysis (Premo™ FUCCI Cell Cycle Sensor)



- S, G2, M phases; - G1/S interphase; - G1 phase

MSCs in AMS became arrested in G1 phase in 48 hrs after encapsulation

Mitochondrial membrane potential by JC1-test

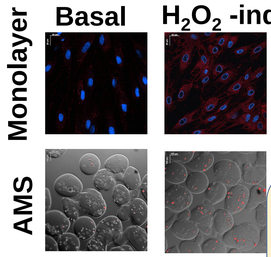


Low mitochondrial membrane potential (λ_{535nm})

High mitochondrial membrane potential (λ_{595nm})

MSCs in AMS had decreased mitochondrial membrane potential

ROS level by Abcam Cellular ROS Kit



RFU/cell	Basal ROS level	H_2O_2 - induced
Monolayer	32,8 ± 5,2*	154,5± 7,8*
AMS	2.32 ± 0.25	11.78 ± 0.44

Encapsulated cells showed lower basal ROS level and higher resistance to hydrogen peroxide.

Conclusion:

Alginate encapsulation results in cell cycle arrest, metabolic activity & ROS level decrease, intracellular pathways modulation, antioxidant defense activation, which increase the resistance of encapsulated MSCs to storage at ambient temperature.

VIABILITY OF HAEMATOPOIETIC STEM CELL PRODUCTS STORED FOR MORE THAN ONE YEAR

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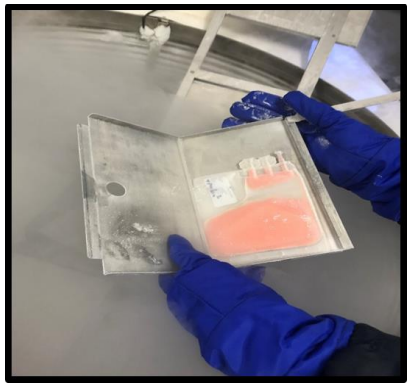
SANBS
Registration No. 2000/026390/08

INTRODUCTION

The Cellular Therapy Laboratory (CTL) of the South African National Blood Service (SANBS) is responsible for the processing and storing of Haematopoietic Stem Cell Transplant (HSCT) products. HSCT products are stored in the vapour phase in liquid nitrogen (LN) freezers. Prior to the release of HSCT products for reinfusion, viability of the storage vials, aliquoted at the time of processing and stored under the same conditions, is performed using flow cytometric methods. HSCT viability release criteria is ≥50% for CD45 and ≥70% for CD34. Discrepancies between vial and product viability are well known, with vial viability often underestimating product viability. HSCT products require rapid infusion post thawing, however, our clinical units are geographically distant from our laboratory, therefore routine product viability testing is not possible in our setting.



METHODS

HSCT products that are no longer required for patient use, are useful for assessing true product viability. This study assessed product viability (CD45 and CD34) of ten redundant HSCT products stored in CTL. Written consent for the use of these products was received from the treating physician.

RESULTS

The HSCT products were older than 1 year with a range of 1-5 years and a mean of 1.7 years. Upon flow cytometric viability analysis, it was found that all products passed the release criteria, with a range of 50-90% for CD45 (mean: 70%) and 96-100% (mean: 96%) for CD34. There was no correlation between age of product and viability of product (correlation coefficient: 0,019309).

Sample No	Days in Freezer	CD34%	CD45%
1	712	96	69
2	933	99	77
3	520	100	67
4	519	100	90
5	383	95	63
6	376	95	72
7	375	87	84
8	1857	94	50
9	343	97	66
10	855	97	56

CONCLUSION

This study showed that HSCT products stored for more than one year still fulfill the release criteria, indicating the robust processing and LN storage procedures in SANBS CTL. Annual testing of samples with increasing ages will be beneficial in determining maximum validated LN storage time periods in this facility.

Submission ID: 97

NUMERICAL MODELING OF CRYOGEN SPRAY EJECTING FROM COMMERCIAL CRYOGUN

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ABSTRACT

❖Cryospray is a method of destructing cancerous lesions occurring on skin. Cryogen is sprayed on the affected area and ablation is achieved through rapid freezing of the cell.

❖An Eulerian- Lagrangian mathematical model is used to simulate the behavior of cryogen spray

❖The validity of the present model has been confirmed with experimental results of evaporating liquid nitrogen spray gushing out form the commercial cryogun.

❖Simulation result predicts 30 mm as most optimized spraying distance on the basis of the area enclosed by lethal front and freezing front.

INTRODUCTION

❖ Cryotherapy is an advance application that utilizes therapeutic effect of low temperature in the area of cancer treatment.
❖ Rapid increment in cancer cases globally and inability of conventional techniques to treat diseases compelled researcher to seek options of cancer treatment.
❖ Cryospray is the type of cryotherapy which deals with superficial tumor.
❖In cryospray cryogen exchanges heat and mass with surrounding compared to cryosurgery in which cryogen circulates in closed cannulas and only exchanges heat with surrounding. It makes the imaging of cryospray a challenging task as compared to cryosurgery
❖Thus, surgeon estimates the dimension of necrotic zone through his experience which makes cryospray more surgeon oriented process rather than comprehensive process.
❖In this perspective present study aim at the development of numerical model that will acknowledge all the parameters involved in the cryospray process.

METHODOLOGY/FORMULATION

❖The Eulerian-lagrangian approach is used to describe the thermo-fluid behavior of liquid nitrogen droplets present in atmospheric air. Continuous phase (atmospheric air) is described by Eulerian framework while dispersed phase (liquid nitrogen droplets) is described using Lagrangian framework.

❖The whole simulation is divided into two parts first atmospheric air flow field is calculated before adding the injection source, after obtaining the stable flow field of atmospheric air liquid nitrogen droplets are introduced into the atmospheric air for further calculation .

❖In simulation, interphase coupling method is used to calculate the influence of energy transfer on the atmospheric air due to the evaporation of nitrogen droplets and vice versa.

BOUNDARY CONDTIONS

❖The temperature of the surrounding air is taken as 300 K.
❖The velocity of surrounding air is taken as 0.1 m/s at inlet condition.
❖The outlet boundary condition are specified as fully developed outflow condition. The exit flow pressure is set at atmospheric pressure.
❖The gravitational acceleration is set as 9.81 m/s^2.
❖Mass flow rate of liquid nitrogen is determined experimentally.
❖Input velocity of droplet is calculated as 5 m/s.
❖Rosin Rammler diameter distribution varies in a range of 20 µm to 100 µm.
❖The exit boundary condition for droplet is set as escape.
❖Both phases air and droplet are two way coupled which means droplets can have effect on surrounding and vice versa.

CONCLUSION

❖Proposed approach explores the phenomena of heat and mass transfer through cryogen spray
❖It has been observed that as the distance from the nozzle exit increases the minimum temperature inside the spray core decreases.
❖Overall decrement of 30 ^{0}C is recorded in the spray core for the spraying distance of 80 mm.
❖Curves of velocity with respect to radial distance flattens as the spraying distance increases.
❖On the basis of area enclosed by lethal front and freezing point it can be concluded that spraying distance of 30 mm is providing the most optimized results

NUMERICAL MODEL USED

❖ Standard k-epsilon turbulent model is used.
❖ SIMPLE algorithm is used for pressure and velocity coupling.
❖ The Rosin Rammler model is used for size distribution of droplet.
❖ Ranz-Marshell model is used to study the evaporation of nitrogen droplets.
❖ Stochastic collision model and coalescence model are also used to acknowledge in effect of breakup and coalescence of droplets.
❖ Spherical drag law is used for calculation of drag force.
❖ Fig .1 and Fig. 2 represents the domain of simulation and mesh respectively.

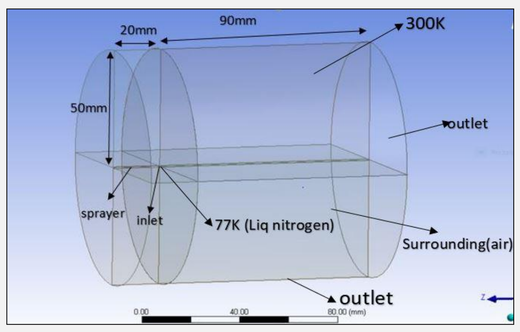

Fig. 1

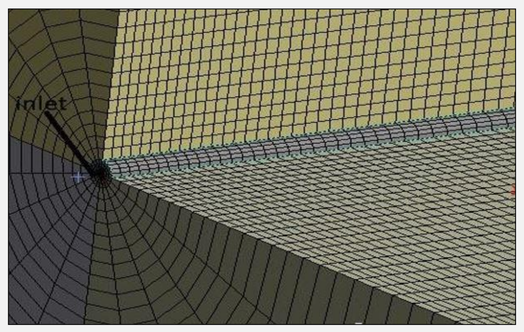

Fig. 2

EXPERIMENTAL VALIDATION

❖ K-Type thermocouple with bead diameter of 0.5mm is used to measure temperature.
❖ Croyogen spray duration of 30 seconds is selected for experiment.
❖ Total 9 thermocouple were used to measure the temperature at discrete locations.
❖ Center of thermocouple and center of nozzle was always maintained symmetrically.
❖ The vertical distance between bead of the thermocouple and nozzle is varied to spray at desired distance.
❖ The numerical results are in good agreement with experimental results as is shown in Fig 3.

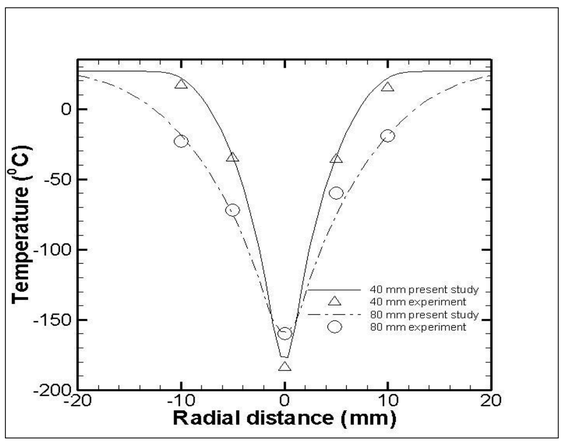

Fig. 3

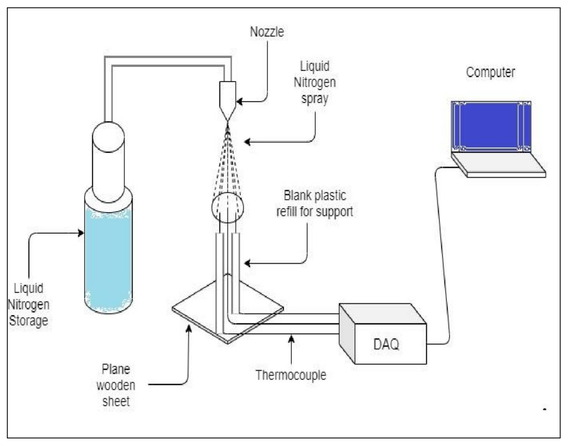

Fig. 4-Schematic representation of experimental setup

RESULTS

❖Fig. 5 represents the temperature contour along the central plane of spray axis
❖ Fig. 6 shows that the cone radius and the temperature of the inner core increases as the distance from inlet increases.
❖ Fig. 7 shows the temperature variation along the spray axis.
❖ Fig. 8 represents the variation in temperature at different spraying distance.

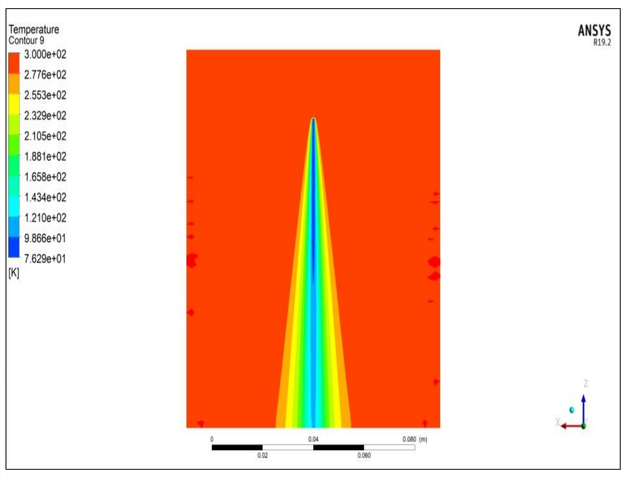

Fig.5

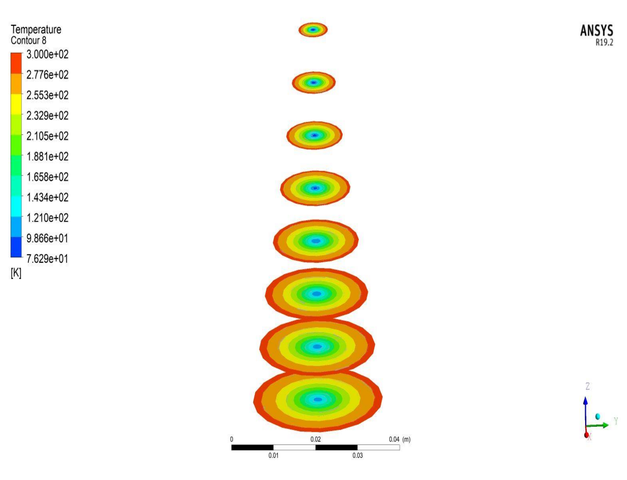

Fig. 6

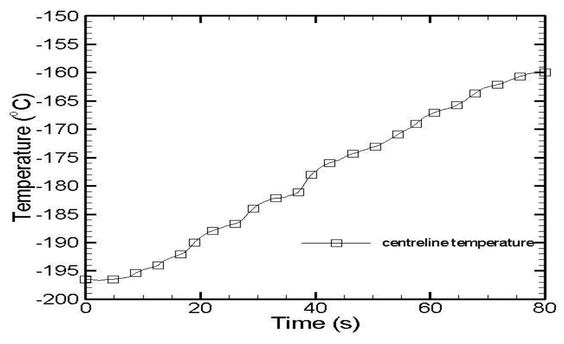

Fig. 7

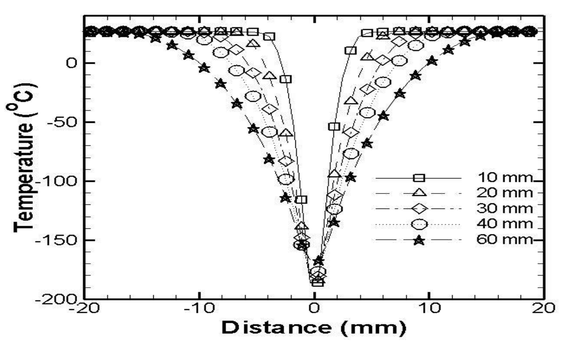

Fig.8

METHODICAL APPROACHES TO CRYOPRESERVATION OF CELLULAR SPHEROIDS

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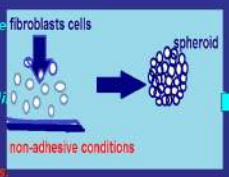
The structure of spheroids (SF) differs from the cell

The search for optimal methods of cryopreservation of spheroids is a topical issue in cryobiology. Numerous cryopreservation protocols for cell suspensions, i.e. single cells, do not solve the problem of cryosensitivity of cells in multicellular spheroids, which have a three-dimensional structural organization and complicated intercellular interactions.

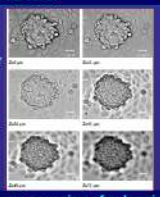
The aim of the study was to determine the filtration coefficients for water molecules and permeability of cell membranes for dimethyl sulfoxide (Me_2SO) into spheroids at different terms of cultivation.

Methods

The study was performed on single cells of the L929 line and spheroids (SF), obtained from this li after 7-14-21 days of cultivation in non-adhesive conditions.



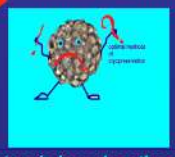
As a result of cultivation of cells of L 929 line, spheroids with a diameter of 80-100 μm were obtained



Layer-by-layer scanning of spheroid after 21 day of cultivation, Without necrotic zones

Using the theoretical calculation proposed in the work, it is possible to determine such parameters

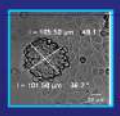
!!! Optimal time of isothermal exposure



!!! Optimal cooling rate

The calculation of permeability coefficients was carried out according to volumetric data and differential equations of Kedem Kachalsky

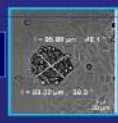
The calculation of these parameters is based on the change of spheroids volume during the time after their contact with 1 M DMSO solution and NaCl solution of different tonicity



V0

Before adding 1M DMSO

After adding 1M DMSO



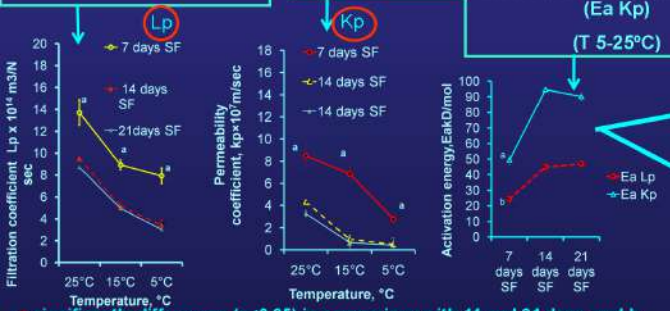
V(t)

Filtration coefficient for H_2O

Permeability coefficient for Me_2SO

Activation energy of water molecules (Ea Lp) and Me_2SO (Ea Kp)

(T 5-25°C)

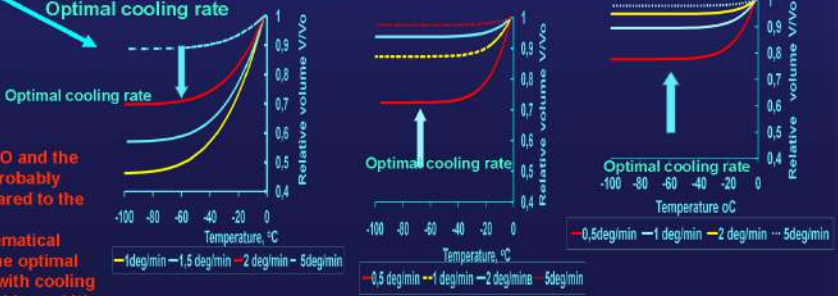


a significantly differences ($p<0.05$) in comparison with 14 and 21 days, and b

The optimal exposure time in 1M DMSO

7 days SF		14 days SF		21 days SF	
5°C	64.8 ±2.9 sec*	5°C	216.5 ± 9.0 sec*	5°C	440.5 ± 11.2 sec*
15°C	51.3 ± 3.6 sec*	15°C	109.1 ± 7.0 sec*	15°C	270.3 ± 2.9 sec*
25°C	43.11 ± 1.7 sec *	25°C	67.7 ± 1.3 sec *	25°C	100.1 ± 3.2 sec*

Optimal cooling rate



Predicted kinetics of change in spheroid volume during freezing depending on various cooling rates: 0.5 , 1 , 2, 5 °C/min.

CONCLUSIONS

It is proved that the exposure time of spheroids in 1M DMSO and the value of the energy of transfer of water molecules and DMSO probably increases 2 times on the 14th and 21st day of cultivation compared to the 7th day of cultivation.

Based on the obtained parameters and physical and mathematical modeling of spheroid dehydration processes during cooling, the optimal cooling rate was determined for 7 days spheroids: 2 deg / min with cooling to -80°C and subsequent immersion in nitrogen; and for spheroids on 14th and 21st day: 1 deg /min to -40°C and subsequent immersion in nitrogen.

REAL-TIME MONITORING OF SKIN TEMPERATURE FIELD DYNAMICS DURING CRYOTHERAPY

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[b]B. Verkin Institute for Low Temperature Physics and Engineering of the National Academy of Sciences of Ukraine, Kharkiv, Ukraine

Introduction

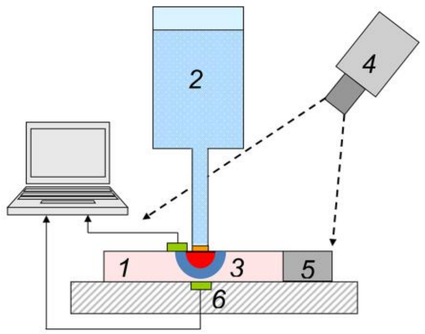
In this study, we assumed that the thermal field parameters in the ice spot on the skin surface, caused by the cryoapplicator, are equivalent to those of underlying tissue frozen volume both during freezing and warming.

Purpose

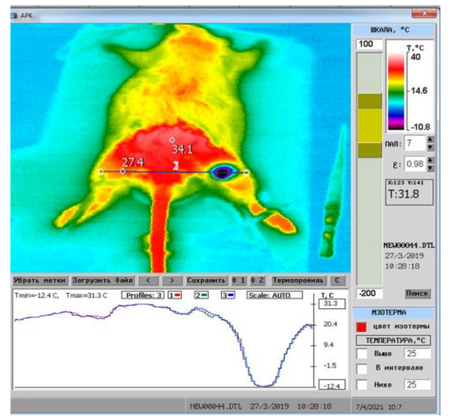
To assess the practical application of infrared thermal imaging for real-time monitoring of freezing and warming of biological tissues in vivo.

Materials & Methods

Cryoablation of the skin in 30 rats was performed with exposure durations of 0.5, 1 and 2 minutes. The liquid nitrogen-cooled contact cryoprobe was used.

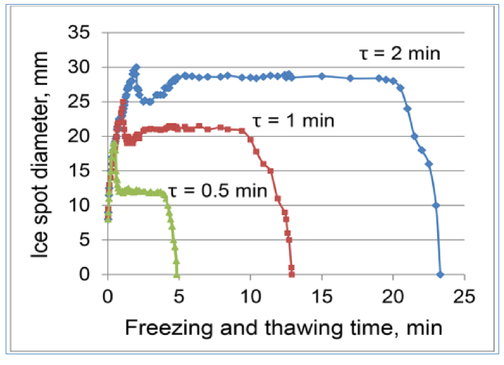


Experimental diagram: 1 – animal, 2 – contact cryoprobe, 3 - ice hemisphere and primary necrosis area, 4 – IR camera, 5 – blackbody, 6 - thermocouple

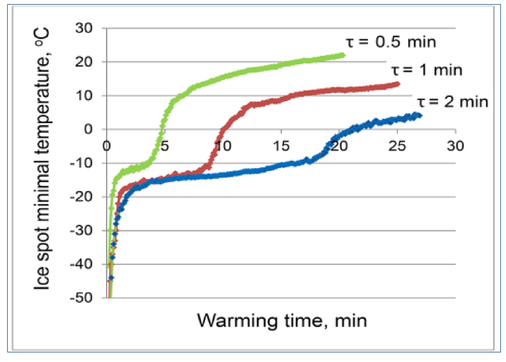


User interface with IR image of animal during warming

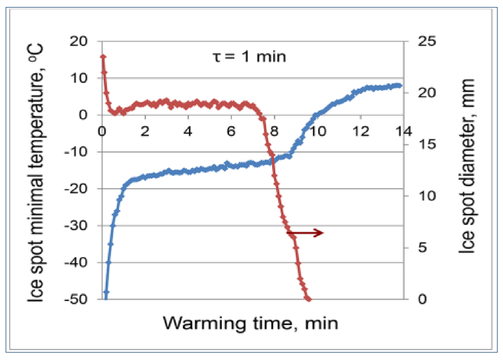
Results, Discussion



Ice spot diameters at various cryoexposure durations and subsequent warming.



Ice spot minimal temperatures during natural warming after various cryoexposure durations.



Ice spot diameter and its minimal temperature after cryoexposure durations of 1 min.

The dynamics of ice spot diameter and its center temperature are similar. The feature of the freezing/thawing cycle includes the specific stages, cryoexposure duration-depended parameters. During natural thawing, the quasi-stable stages (QSS) are present in both dynamics, apparently associated with structural ice rearrangements in the frozen tissue.

Ice spot parameters measured during thawing for animal groups under study.

Measured parameter	Group 1	Group 2	Group 3
Max. diameter of ice spot, mm	18.54 ± 1.00	23.66 ± 1.29*	28.35 ± 1.06*#
Max. diameter of necrosis spot, mm	11.91 ± 0.82	15.10 ± 1.15*	18.30 ± 0.82*#
Diameter of ice spot in QSS, mm	11.74 ± 0.93	19.52 ± 2.05*	25.72 ± 1.18*#
QSS start time, min	0.87 ± 0.20	2.13 ± 0.30*	1.75 ± 0.26*
QSS duration (on diameter), min	2.51 ± 0.47	5.13 ± 0.32*	10.83 ± 1.33*#
QSS duration (on temperature), min	2.37 ± 0.33	5.04 ± 0.56*	10.32 ± 1.52*#
Temperature at QSS beginning, °C	-12.74 ± 0.73 -	16.64 ± 0.66*	-13.95 ± 1.31#
Full thawing time, min	4.18 ± 0.35	10.89 ± 0.86*	20.18 ± 2.18*#

The asterisk (*) indicates significant difference between the group parameter compared analogous parameter of the group 1,the # - of the group 2.

To conclude we have shown that infrared imaging can be used for real-time monitoring of skin temperature field dynamics during cryotherapy, including the examination of the necrotic and cryoscopic isotherm positions.

Acknowledgements

This work was supported by the National Academy of Sciences of Ukraine funding by the grant 0121U108515

THE EFFECT OF HYPOBIOSIS DURING E.COLI INFECTION

NATIONAL UNIVERSITY OF LIFE AND ENVIRONMENTAL SCIENCES OF UKRAINE

Aim of research

As artificial sleeping is increasingly considered for use as a stand-alone therapeutic agent, *our study was to examine* the effect of its administration on *E. coli* infected animals and to elucidate the biochemical parameters of the body at different stages of infection.

Methods and materials

The experiments were performed with 2-months rats, thay were led in for 3 hours in the hypobiosis (Bakhmetev-Jaya-Anjus method with lowering the animals' body temperature);

We have used a sealed chamber with a volume of 3 dm3 at temperature 3–5 ° C.

Infection's stages were initial symptoms (I), progressive symptoms (II) and total depletion of the body (III stage);

We evaluated the antioxidant status and the oxidative status in the liver tissues and the pattern of redistribution of protein fractions in the blood.

Conclusions

- SOD and catalase activities of biological samples have shown that hypobiosis stimulates the body to fight E. coli (I and II stages) by activating SOD and catalase in liver tissues on the principle of additive synergism.
- Analyzing the glutathione antioxidant system of the liver of a sick body in conditions of artificial sleep, it was found that the dynamics of its indicators in general duplicates the picture of SOD and catalase in the same conditions for I and II stages.
- While the introduction of hypobiosis in rats with E. coli infection on stage III is accompanied by depletion of glutathione resource in liver tissues and low glutathione reductase activity.
- The effect of hypobiosis is manifested by a decrease in the level of MDA in the liver of patients at all stages of E. coli infection.
- The proteinogram showed an increase in the level of γ-globulins and a decrease in the level of albumin.
- The obtained results indicate a real prospect of using a 3-hours of hypobiosis for the treatment of E. coli infection at all stages of the disease as a modern cryogenic agent.

Indicators of antioxidant activity of liver tissues in rats with Escherichia coli in conditions of hypobiosis

Indicator	A group of animals							
	Control	In a state of hypobiosis (experimental control)	Escherichia coli infection in stage I	Escherichia coli infection in stage I + Hypobiosis	Escherichia coli infection in stage II	Escherichia coli infecion in stage II + Hypobiosis	Escherichia coli infection in stage III	Escherichia coli infection in stage III + Hypobiosis
SOD µmol / min × mg protein	12,024 ±1,22	15,51 ±1,34	13,03 ±1,09	20,00 ±2,05*	21,60 ±2,67	24,02 ±2,09*	9,62 ±0,87	18,21 ±1,15*
Catalase µmol H2O2 / min × mg protein	65,00 ±6,67	92,95 ±8,70	70,01 ±6,45	99,01 ±8,08*	60,00 ±5,95	100,20 ±9,33*	66,00 ±6,09	70,01 ±5,09*
The content of reduced glutathione (µmol / g tissue)	0,365 ±0,004	0,360 ±0,004	0,355 ±0,003	0,238 ±0,003 #,**	0,274 ±0,013	0,170 ±0,012 #,**	0,189 ±0,012	0,109 ±0,0118, **
Glutathione peroxidase activity (nmol H2O2 / min × mg protein)	10,050±1,005	9,817± 1,002	10,001±0,976	7,919± 0,523	12,581± 1,004#,**	8,125± 0,766	14,572± 1,211#,**	9,512± 0,563
Glutathione reductase activity (µMol / min × mg protein)	0,590± 0,061	0,575± 0,062	0,610± 0,015	0,408± 0,013#,**	0,679± 0,052#	0,599± 0,060	0,413± 0,039#	0,303± 0,029#,**

** - the difference is significant compared with the activity of SOD and catalase of rats with Escherichia coli infection at the appropriate stage of disease development and indicators of healthy animals in a state of hypobiosis, P≥0.05; # - the difference is significant compared to the reduced glutathione content/glutathione peroxidase of healthy animals, ** - the difference is significant compared to the reduced glutathione content/glutathione peroxidase of animals in a state of hypobiosis, P≥0.05.*

Phase behavior of sucrose-containing cryoprotective solutions at temperatures below 0 °C

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Ukraine

Introduction

Sucrose is often used both as a primary cryoprotectant and in complex cryoprotective media. In particular, sucrose is a component of many media for cryopreservation of mammalian embryos by vitrification.

Purpose

to study low-temperature phase transitions in sucrose-containing solutions of glycerol (Gl), 1,2-PD, 1,3-PD, ethylene glycol (EG), and Me_2SO.

Materials and methods

Low temperature differential scanning calorimetry (DSC)

DSC thermograms were recorded at the heating stage (0.5 grad/min) after rapid cooling of the solutions by immersion into liquid nitrogen (~200°/min).

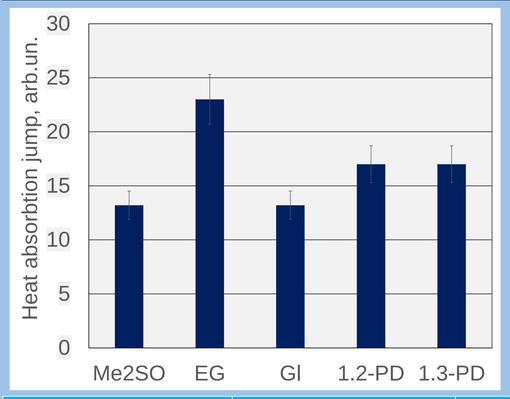
Preparation of cryoprotectant solutions

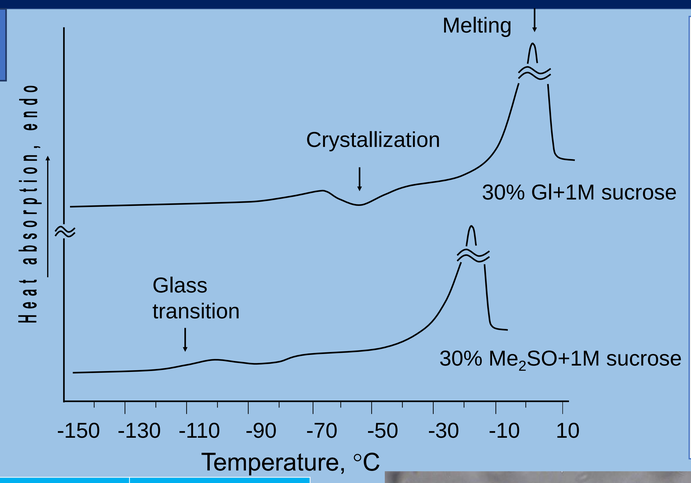
Cryoprotectant solutions (30% concentration) were prepared with Dulbecco's nutrient medium supplemented with sucrose (1 M).

Cryopreservation of mouse embryos

2-cell and 8-cell mouse embryos in cryoprotective medium were vitrified by immersion into liquid nitrogen using plastic straws and preserved 3-7 days. Thawing was performed in water bath (38°C). To remove the cryoprotectant there was used a 10-min equilibration in 0.5M sucrose solution. Then the embryos were three times washed-out with physiological medium, transferred into CO_2 -incubator for culturing. Rate of embryo viability was estimated by their developmental capacity to the stage of extended blastocyst.

Results

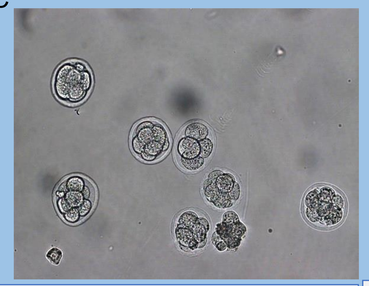




Immediately after glass transition, for all investigated solutions, crystallization completed at the heating stage, which indicated a low stability of the amorphous state. In solutions of Me2SO and EG, the lowest glass transition, crystallization and melting temperatures were recorded. After the addition of sucrose no crystallization of eutectic was recorded in any of the studied cryoprotectant solutions.

Samples	T_g, ºC	T_c, ºC	T_m, ºC
Me_2SO	-108.2±0.5	-85.3±0.5	-34.6±0.5
EG	-109.1±0.5	-86.9±0.5	-25.8±0.5
Gl	-76±0.5	-54±0.5	-11.7±0.5
1.2-PD	-72.2±0.5	-51.6±0.5	-13.9±0.5
1.3-PD	-83±0.5	-62.1±0.5	-15.1±0.5

[a] -25 min of 2- cell embryo exposure in Dulbecco's physiological medium at room temperature; [b] – statistically significant difference compared to the control, p<0.05



8-cell mouse embryos after vitrification using Gl-sucrose medium

2-cell mouse embryos after vitrification using EG-sucrose medium

Developmental stage	Groups	Cryopreservation medium	Number	Exposure time in cryoprotectants	Number of embryos, approaching blastocyst stage,(n)%
2-cell	control	30% EG + 0.7 M of sucrose	52[a]	–	(47) 90,4±4,1
8-cell		30% Gl + 0.7 M of sucrose			(39) 90,2±2,2
2-cell	I	30% EG + 0.7 M of sucrose	58	1,5 min	(52) 89,7±4,0
8-cell		30% Gl + 0.7 M of sucrose			(31) 84,9±4,3
2-cell	II	30% EG + 0.7 M of sucrose	53	3 min	(38) 75,5±6,3
8-cell		30% Gl + 0.7 M of sucrose			(23) 56,8±6,7[b]
2-cell	III	30% EG + 0.7 M of sucrose	74	1,5 min	(31) 55,4±7,6[b]
8-cell		30% Gl + 0.7 M of sucrose			(65) 80,2±6,2

Conclusions

the addition of sucrose to cryoprotective solutions leads to an increase in the glass transition temperature and the disappearance of crystallization and melting of eutectic compositions.

ADDITION OF FERULAGO ANGULATA EXTRACT TO FREEZING EXTENDER FOR GOAT SPERM CRYOPRESERVATION

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Introduction

During cryopreservation, goat spermatozoa are exposed to different types of physical, chemical, osmotic, and oxidative stresses that have adverse effects on the quality and fertility of sperm. Since, providing a cryomedium supplement for freezing sperm can enhance the artificial insemination efficacy and afterward, the reproductive performance of goats. Despite the antioxidant properties of *Ferulago angulata* extract (FAE), the effect of this natural antioxidant as an additive for the semen extender has not been studied.

Aim

In this study, we evaluated the effect of FAE supplementation into freezing extender on the cryopreserved goat semen quality and fertility ability after thawing.

Methods

In experiment 1, the treatments consist of basic extender containing different concentrations (0.00, 0.002, 0.005, and 0.01%, w/v) of FAE. After determination of the potential effective concentration of FAE using assessment of the sperm motility, In experiment 2, semen samples (15 ejaculates/3 goats) were pooled, diluted with Bioxcell® extender, and supplemented with 0.002% FAE. Control diluent contained no additives. Following equilibration, the straws were exposed to liquid nitrogen (LN2) vapor and then plunged into LN2. After thawing, sperm quality and fertility were evaluated.

Results

Our results showed that the sperm motility and plasma membrane integrity after thawing significantly improved by FAE compared to the control group. The sperm viability was not different between the two groups. ROS production level significantly decreased by FAE compared to the control. FAE supplement improved the hatched blastocyst rate in embryos derived from frozen/thawed goat sperm compared with the control group. No significant differences were observed in cleavage and blastocyst rates of embryos among FAE and control groups.

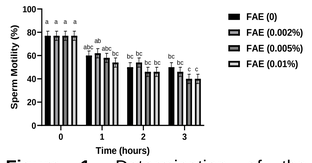

Figure 1. Determination of the potential effective concentration of FAE for goat semen cryopreservation using evaluation of the sperm motility

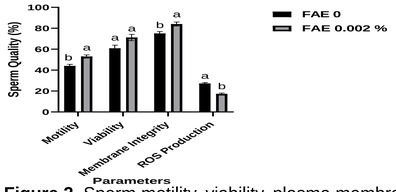

Figure 2. Sperm motility, viability, plasma membrane integrity, and ROS production in frozen/thawed goat spermatozoa with extender supplemented with FAE

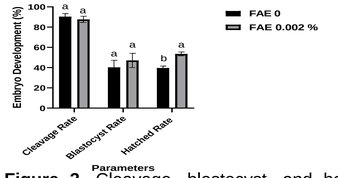

Figure 3. Cleavage, blastocyst, and hatched blastocyst rates in embryos derived from frozen/thawed goat spermatozoa with extender supplemented with FAE

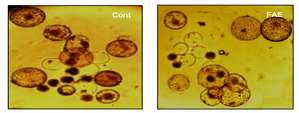

Figure 4. Representative images of embryo development at 8 days post fertilization of oocytes with thawed goat spermatozoa cryopreserved with extender supplemented with FAE.

Conclusion

As an overall conclusion of this study, the addition of FAE (0.002%, w/v) to the freezing extender improved goat sperm quality and fertility after the freeze-thaw procedure. Hence, FAE as a natural antioxidant can increase sperm cryotolerance and post-thaw persistence.

Acknowledgments

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EFFECTS OF RUTIN ON THE QUALITY OF ROOSTER SPERM DURING CRYOPRESERVATION

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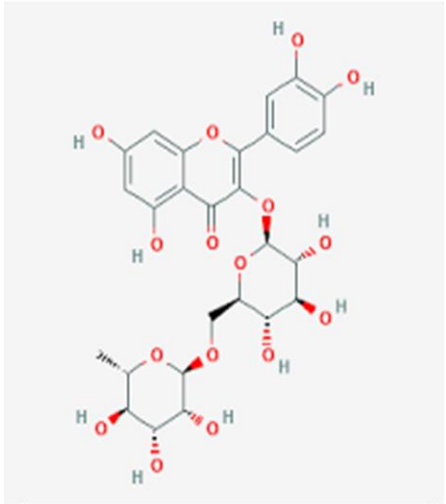
Department of Animal Science, College of Agriculture, University of Tabriz, Tabriz, Iran;

BACKGROUND

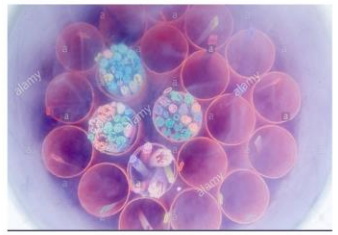
Rutin is known as a plant pigment (flavonoid) found in green tea, passion flower, buckwheat, and apple. In this experiment, rutin has been selected because of the interesting findings of flavonols in genotoxicity tests. It is demonstrated that flavonols show a double-edge actions, because they can perform as prooxidants or antioxidants, depending on concentration. The strong antioxidative capacity of rutin has been confirmed by several experiments, mainly for outstanding scavenging activity. The purpose of the current study, was to estimate the effect of different levels of rutin on post-thawed rooster semen quality.



RUTIN

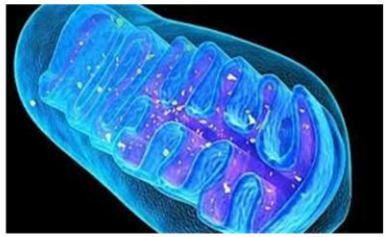
MATERIALS AND METHODS

Ejaculates were collected using the dorso-abdominal massage method and collection was always accomplished by the same person and with the same conditions. After collection, the ejaculates were then transferred to the laboratory by a thermal flask containing water at a temperature 37 ° C for primary evaluation. To reduce individual differences and achieve satisfactory sperm for analysis, in each replicate, the ejaculates of the ten roosters were concisely inspected and ejaculates with ≥ 300 × 106 spermatozoa/mL ≥ 80% motility and, ≥ 90% normal morphology were then pooled. The diluted semen was gradually cooled to 4 ° C and then cryopreserved in 0.25 mL French straws. Frozen straws were thawed at 37 ° C for 30 s in a water bath, for post-thawed sperm evaluation.



RESULTS

Rutin at level 0.6 mM resulted in the highest total and progressive motilities percentages, in comparison to other treatments ($P < 0.05$). Our results revealed that rutin at level 0.6 mM led to higher GPx, mitochondria activity, and membrane integrity in comparison to the control group ($P < 0.05$).



CONCLUSION

It can be deduced that addition of rutin at level 0.6 mM improved the post thawing quality and oxidative variables of rooster semen.

KEYWORDS

cryopreservation; sperm; rooster; extender

Changes in the redox state of cytochromes in mouse embryos during cooling with different cryopreservation protocols

CRYO ❄ 2021 (online meeting)

by K.A. Okotrub[1], V.I. Mokrousova, S.Ya. Amstislavsky[2], and N.V. Surovtsev[1]

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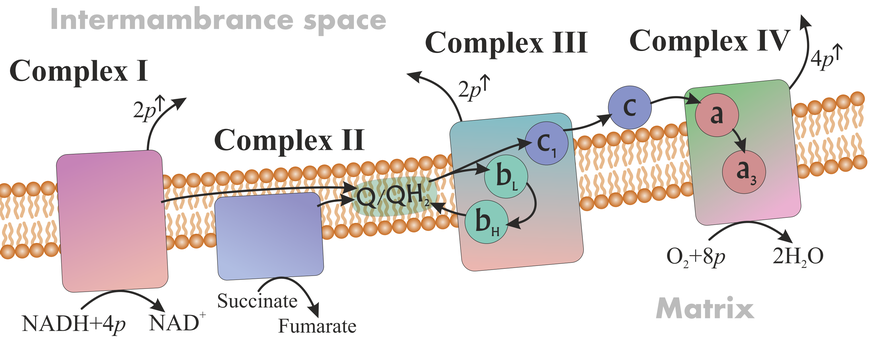
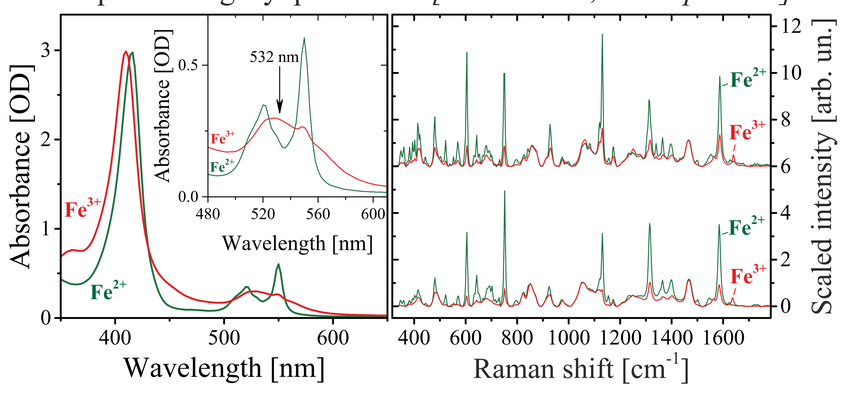
Electron Transport Chain



Introduction

Cytochromes are haemoproteins that participate in the work respiratory electron transport chain (ETC), the fundamental process of cellular respiration. The intensity of resonance Raman lines of cytochrome haem depends on the cytochrome redox state. We applied this effect to characterize the activity of respiratory electron transport chain (ETC) in mouse early embryos and yeast cells during freezing.

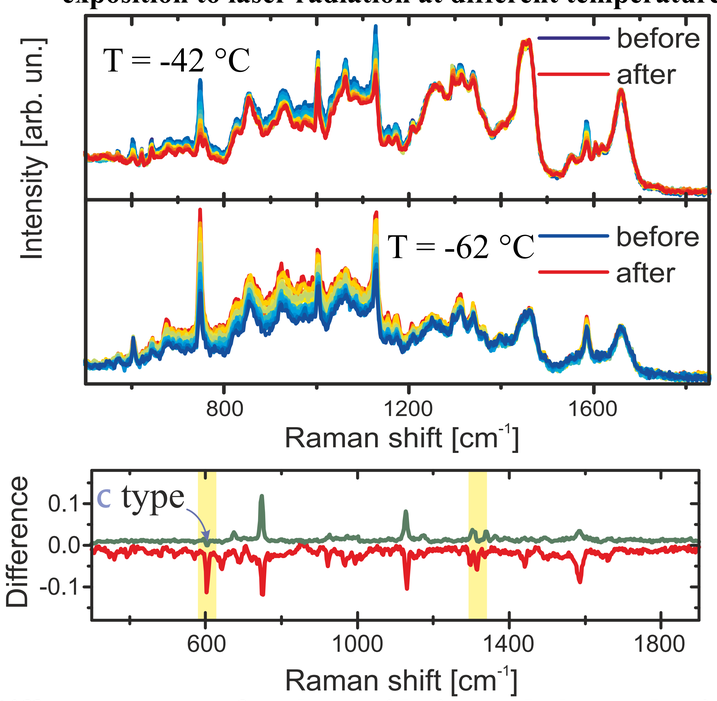
Green light radiation induces the formation of reactive oxygen species in biological cells resulting in oxidative stress and oxidation of cytochromes. Oxidation process leads to photobleaching of cytochrome Raman lines. Here we applied this effect to characterize the activity of ETC in biological cells during freezing and compared the cytochrome redox state of cytochromes in embryos cooled to –170°C using different cryopreservation protocols: slow program freezing, vitrification with penetrating cryoprotectants, vitrification without penetrating cryoprotectants *[Jin & Mazur, Sci. Rep. 2015]*.



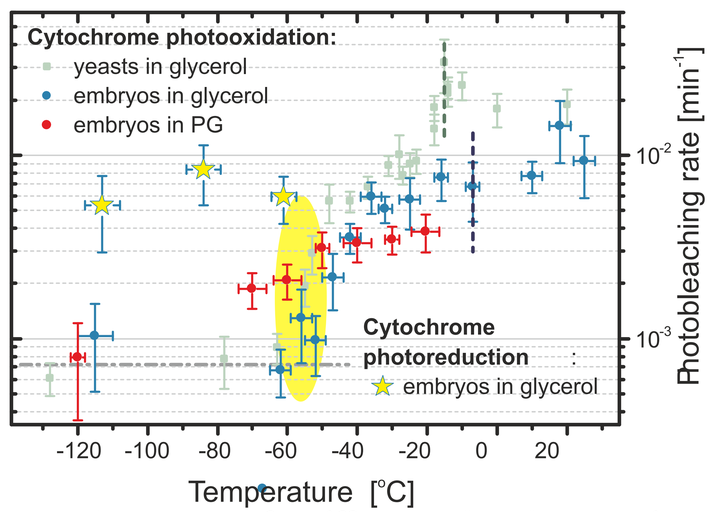
For c & b type cytochromes, the intensity of the Raman line at 600 and 750 cm^{-1} in Fe^{2+} state is ~10 times greater than in Fe^{3+}.

Results

Examples of mouse embryo Raman spectrum evolution during exposition to laser radiation at different temperatures.

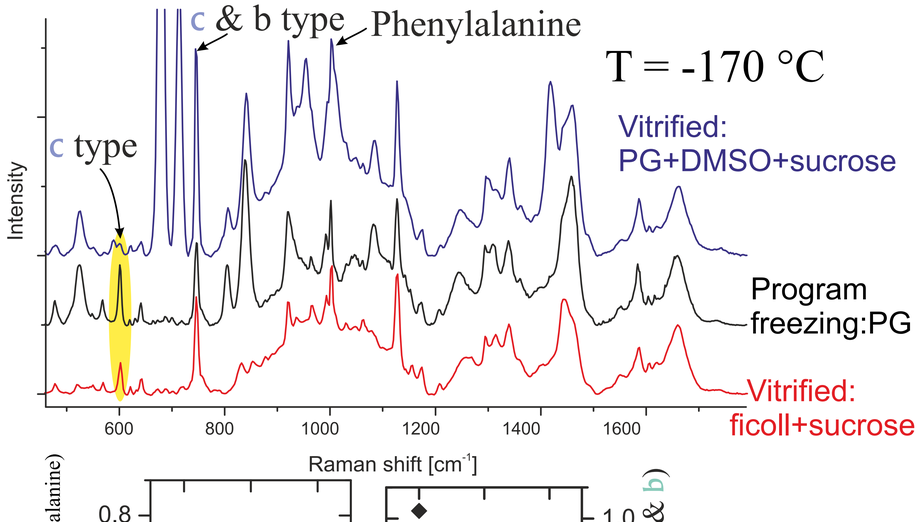


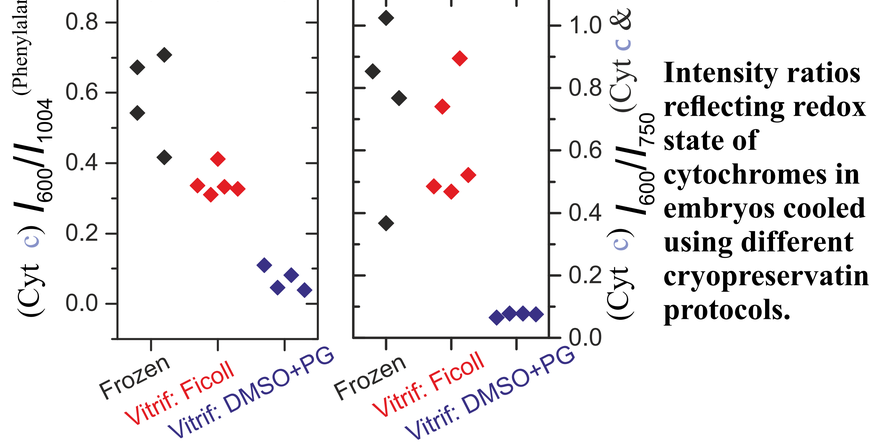
Difference spectra for photooxidation and photoreduction of cytochromes in mouse embryos during cooling



Temperature dependences for different redox photoreactions rates. The yellow ellipse marks an abrupt slowdown below -50°C.

Raman spectra of frozen and vitrified mouse embryos measured at -170°C.





Intensity ratios reflecting redox state of cytochromes in embryos cooled using different cryopreservatin protocols.

Conclusions

- Changes in the photobleaching rate and increase of cytochrome Raman lines intensity observed in yeast cells and mouse embryos indicate on the changes in ETC state at about -50°C.
- Difference spectra analysis revealed photoinduced reduction of b type cytochromes and oxidation of c type cytochromes in mouse embryos at temperatures below -50°C. This effect can be interpreted as an oxidant-induced reduction effect indicating on downregulation of bc1 complex of the ETC in frozen cells.
- We observed that embryos vitrified with penetrating cryoprotectants demonstrate the lowest concentration of cytochrome c in reduced redox state.

Okotrub, K. A., & Surovtsev, N.V. (2015). Redox state of cytochromes in frozen yeast cells probed by resonance Raman spectroscopy. Biophys J, 109, 2227-2234.
Okotrub, K.A., & Surovtsev, N.V. (2018). Effect of glycerol on photobleaching of cytochrome Raman lines in frozen yeast cells. Eur Biophys J, 47:655–662
Sazhina E.A., Okotrub K.A., Amstislavsky S.Y., Surovtsev N.V. (2019). Effect of low temperatures on cytochrome photoresponse in mouse embryos. Arch. Biochem. Biophys. 669:32-38

Cryopreservation of domestic cat preimplantation embryos: effects of in vitro exposure to linoleic acid

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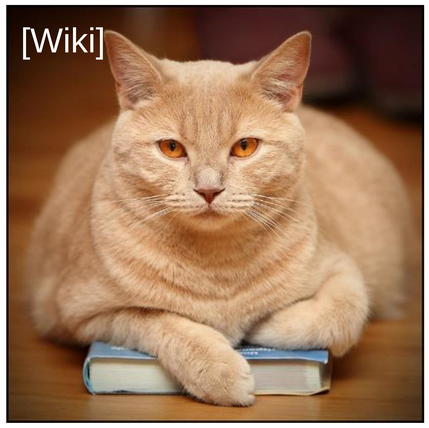
[1] Institute of Cytology and Genetics, Novosibirsk, Russia; [2]Institute of Automation and Electrometry, Novosibirsk, Russia; [3]Novosibirsk State University, Novosibirsk, Russia

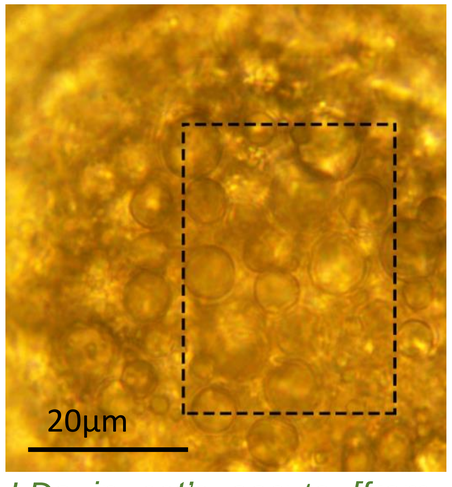
Introduction *Felidae* species are the focus of wildlife conservation activity. The domestic cat (*Félis silvéstris cátus*) is considered as a model species for the applying technologies of Genome Resources Bank to wildlife felids



Félis silvéstris cátus

Embryos of *Carnivora* species are characterized by high lipid content; for such embryos rich in lipid droplets (LDs), tolerance to freezing and cryopreservation (CRYO) is low. The problem can be solved by a change in the composition of intracellular lipids.

The aim This work is aimed to study the effect of linoleic acid (LA) in vitro exposure on the intracellular lipids and the onset of their phase transition (T*) during freezing, as well as the efficiency of embryo cryopreservation.



LDs in cat's oocyte [from Ranneva et al.,2020]

Results

The addition of LA to the medium during in vitro culturing (IVC) of cat embryos did not influence the overall number of lipids assessed by fluorescent intensity of the Nile red (Fig.1). However there was found an increasing of the intracellular lipid unsaturation degree and a decrease in the onset of lipid T* as compared to control (Fig.2).

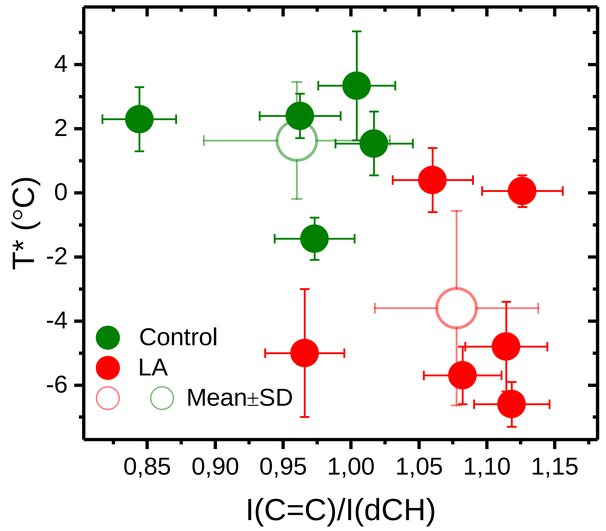


Fig.2. Dependence of the lipid's T* on the level of their unsaturation.

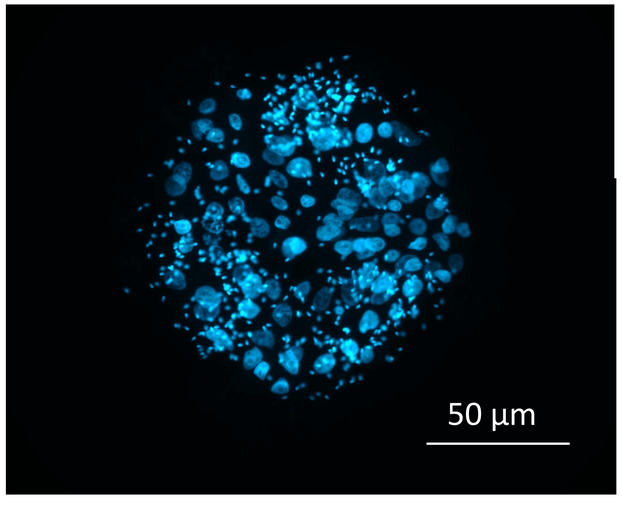


Fig.3. Blastocyst from LA group, after CRYO and the subsequent IVC (95 h). DAPI staining.

An improvement of the development of embryos after cryopreservation was demonstrated: the increased number of interphase nuclei were observed in blastocysts cultured with LA before CRYO as compared with controls. Besides, more advanced blastocysts stages were found in LA group (Table 1, Fig.4).

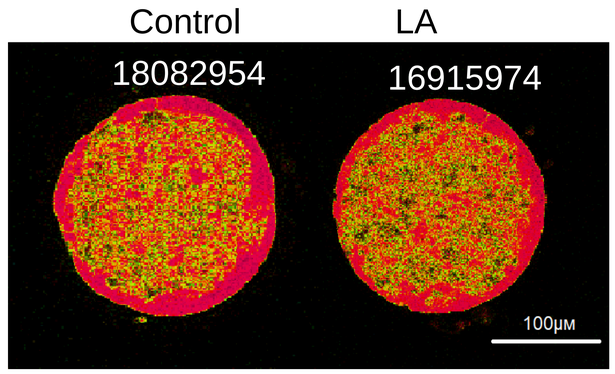


Fig.1. Optical slices of cat's embryos labeled with Nile Red.
CLSM. Photon count mode

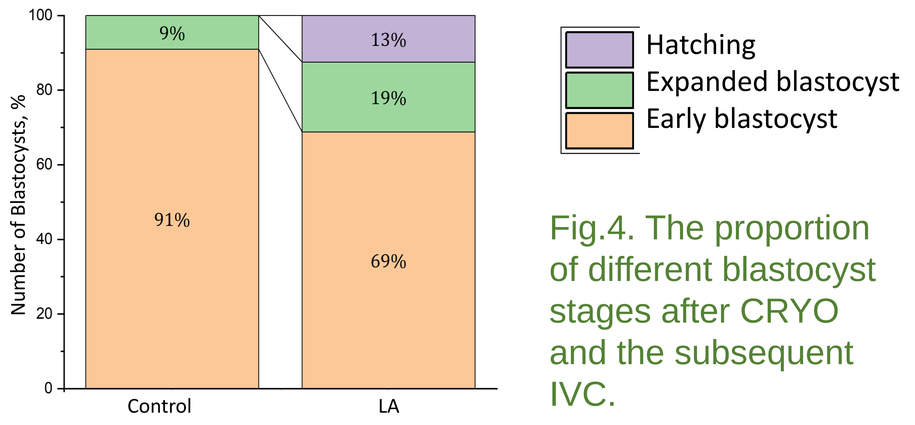


Fig.4. The proportion of different blastocyst stages after CRYO and the subsequent IVC.

Table 1. No. of interphase nuclei after cryopreservation

Group	Morula	Blastocyst
Control	36.9±3	62.6±5.4
LA	27.7±2.7*	91.8±10.7**

Conclusion

The addition of LA to the culture medium during IVC led to an increased unsaturation of intracellular lipids of in cat' embryos. The observed change in the degree of lipid unsaturation led to a decrease in their T*, and caused an improvement of the embryo development after CRYO.