Standardizing the cryopreservation procedure of European perch semen for the development of consistent procedures and future implementation of cryopreservation technology in commercial hatcheries

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JUSTIFICATION

The peculiar characteristic of female reproductive system is that European perch eggs are included within a large, cylindrical gelatinous strand called ribbon. This creates some difficulties in the *in vitro* fertilization procedures at practical conditions.

AIM

The aim of this study was to evaluate the effects of cryopreservation of semen on fertilization rates of entire ribbon of egss obtained from female.

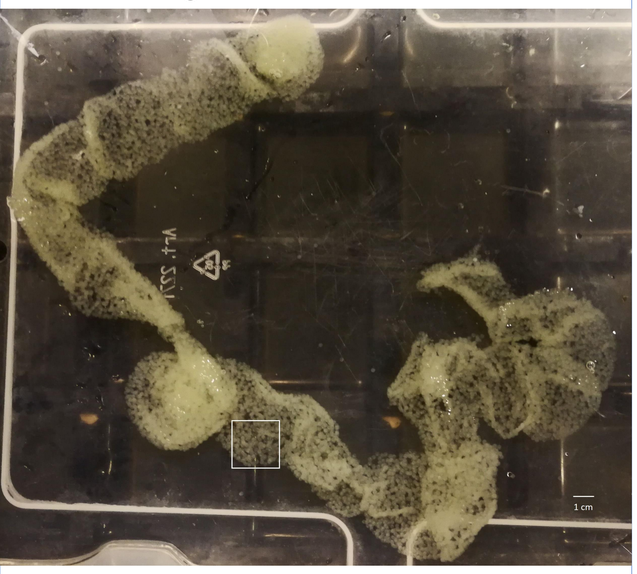
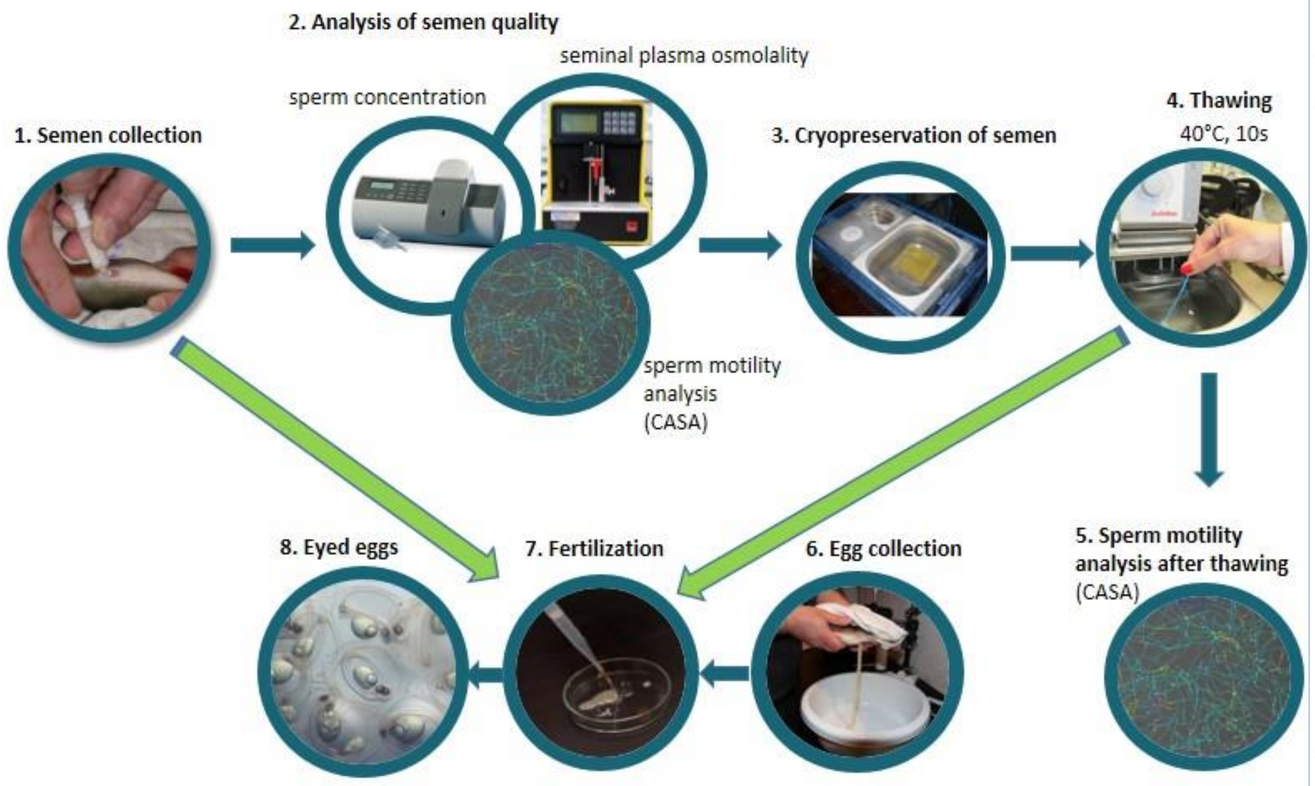


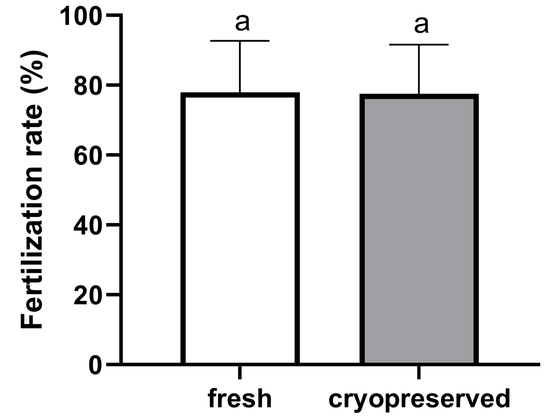
Photo of the entire ribbon obtained from female. The scrap used for fertilization with fresh semen is marked with a white square.

EXPERIMENTAL DESIGN



RESULTS

The fertilization rates of whole ribbons of eggs using cryopreserved semen were high (77%) and did not differ significantly from values recorded for fresh semen (78%).



The effect of sperm cryopreservation on fertilization rates of whole ribbons of eggs determined at eyed eggs (n=7). Results are expressed as mean ± SD.

CONCLUSIONS

Using standardized procedure, it is possible to obtain high motility and fertility values, similar to fresh semen. Cryopreservation procedure of the perch semen presented in this study may be implemented in commercial hatcheries.

ACKNOWLEDGEMENTS

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INVESTIGATION OF THE ANTIOXIDANT CAPACITY OF DITHIOTHREITOL AND GLUTATHIONE ON LARGE WHITE BOARS SEMEN DURING CRYOPRESERVATION.

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INTRODUCTION

Cryopreservation is a process that increases the production of reactive oxygen species (ROS), which in turn, alters the redox state of the cells, eventually leading to stress response. Oxidative stress is an important factor in cryo-injury which causes the lipid peroxidation, loss of sperm motility and viability. Therefore, supplementation of cryopreservation extenders with antioxidants provides both cryoprotective effects and reduces oxidative stress in sperm quality, hence minimizing the detrimental effect of ROS and improving quality of post-thaw sperm.

AIM

The study aimed to compare antioxidant capacity of Dithiothreitol (DTT) and Glutathione (GSH) following cryopreservation of Large White boars semen.

MATERIALS AND METHODS

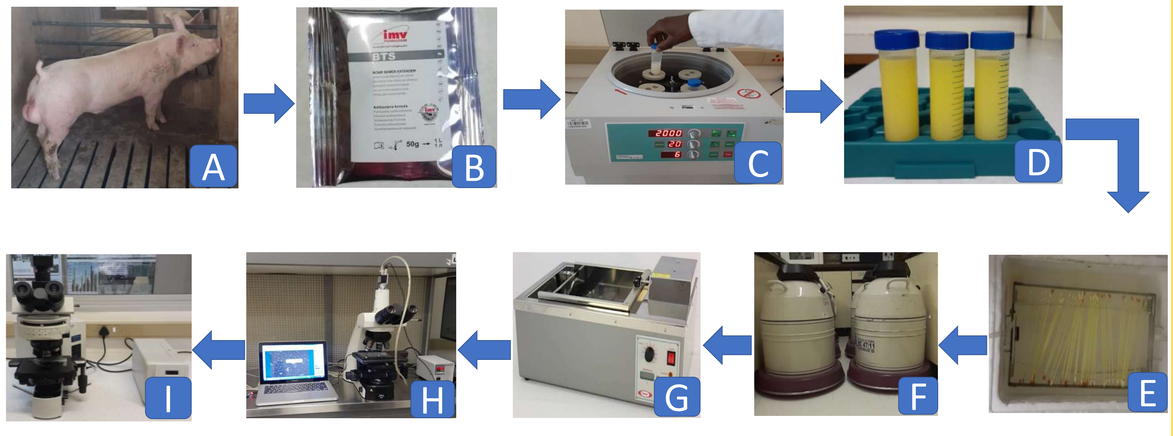

Figure 1: (A) Large White boar, (B) Beltsville Thawing Solution, (C) Centrifuge machine, (D) Egg yolk freezing extenders, (E) Liquid nitrogen vapor, (F) Liquid nitrogen tanks, (G) Water bath (H) Computer aided sperm Analysis system, (I) The fluorescent microscope.

RESULTS

Table 1: Characterization of sperm motility and velocity rates of frozen-thawed Large White boar supplemented with antioxidants.

Boar semen	TM (%)	PM (%)	RAP (%)	VCL (μm/s)	VSL(μm/s)	VAP (μm/s)	LIN (%)	STR (%)	ALH (μm/s)	BCF (Hz)
Raw semen	97.3±2.7^{a}	51.7±10.7^{a}	39.4±14.1^{a}	109.3±17.9	27.1±3.6^{a}	61.3±8.6	26.9±4.5^{a}	46.9±5.3	2.6±0.4^{c}	26.2±1.4^{a}
Control	32.1±6.7^{b}	19.1±7.0^{b}	17.0±6.6^{b}	111.6±18.3	22.8±4.7^{b}	50.7±11.2	21.3±3.1^{b}	44.7±4.8	3.2±0.4^{ab}	17.7±5.2^{b}
5μM DTT	25.7±6.9^{cd}	16.5±5.7^{bc}	13.4±4.9^{b}	106.8±21.5	24.8±5.4^{ab}	53.6±17.1	23.4±3.9^{ab}	47.8±5.9	3.2±0.7^{b}	18.4±5.4^{b}
5μM GSH	26.0±9.4^{bc}	15.2±5.7^{bc}	12.4±6.0^{b}	114.7±25.2	24.6±4.2^{ab}	53.6±14.2	23.0±5.4^{b}	47.1±8.8	3.3±0.6^{ab}	16.7±4.1^{b}
2.5μM GSH + 2.5μM DTT	19.5±6.4^{d}	10.4±4.6^{c}	11.9±9.1^{b}	123.0±24.8	23.1±3.9^{b}	55.4±16.3	22.0±3.1^{b}	47.1±8.1	3.7±0.6^{a}	16.4±5.5^{b}

$^{a-c}$ Values with different superscripts within the column are different statistically (P< 0.05).

Table 2: Characterization of sperm viability, morphology and acrosome integrity of frozen-thawed Large White boar supplemented with antioxidants.

Boar semen	Viability (%)		Acrosome integrity (%)		Live sperm abnormalities (%)			
	Live	Dead	Reacted acrosome	Non reacted acrosome	Head defects	Tail defects	Proximal droplets	Distal droplet
Raw semen	82.8±8.3^{a}	13.7±8.3^{c}	73.3±12.1^{a}	26.7±12.1^{b}	0.5±0.7	1.8±0.9	0.7±0.7	0.3±0.5
Control	34.9±6.5^{b}	62.5±6.4^{b}	25.2±5.4^{b}	74.8±5.4^{a}	0.5±0.7	1.2±0.7	0.2±0.4	0.3±0.5
5μM DTT	29.8±5.2^{b}	67.4±5.8^{b}	22.4±13.2^{b}	77.6±13.2^{a}	0.2±0.4	1.7±0.8	0.5±0.5	0.4±0.5
5μM GSH	29.4±6.4^{b}	68.4±6.8^{b}	24.4±11.7^{b}	75.6±11.7^{a}	0.5±0.5	1.1±0.7	0.3±0.5	0.3±0.5
2.5μM DTT + 2.5μM GSH	21.7±6.9^{c}	76.0±7.5^{a}	24.5±14.1^{b}	75.5±14.1^{a}	0.3±0.5	1.70.7	0.5±0.7	0.1±0.3

$^{a-c}$ Values with different superscripts within the column are different statistically (P< 0.05).

CONCLUSION

In conclusion, supplementation of DTT + GSH to freezing extender reduced sperm total motility, progressive motility and viability. However supplementation of GSH, DTT and GSH + DTT did not have an effect on sperm velocity parameters, abnormalities and acrosome integrity.

ACKNOWLEDGEMENT

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Direct (alkaline and neutral Comet and TUNEL) but not indirect methods (SCD and SCSA) relate the percentages of sperm with fragmented DNA to chromatin damage in cryopreserved boar sperm

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INTRODUCTION

Sperm DNA damage (SDF) has been assessed over the years as a biomarker for sperm quality, but the low incidence found in livestock animals such as pig leads to concerns about its potential value. The comparison of different tests is important to understand their implication in fertility.

OBJECTIVE

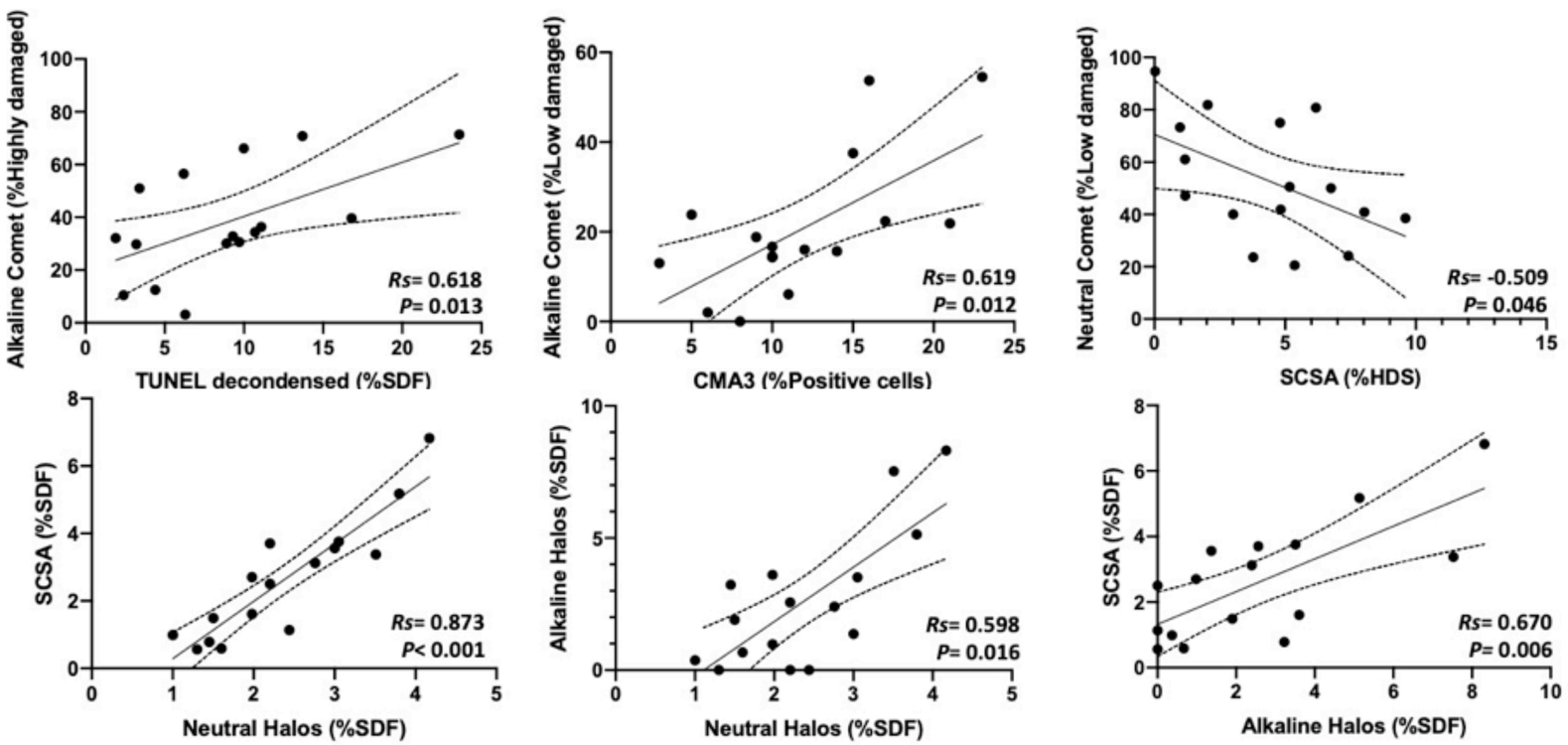
To analyze eight methods assessing chromatin damage in pig sperm.

MATERIAL AND METHODS

Cryopreserved sperm samples from 16 boars were evaluated through: TUNEL, TUNEL with previous decondensation, SCSA, alkaline and neutral sperm chromatin dispersion (SCD) tests, alkaline and neutral Comet assays, and chromomycin A3 test (CMA3). In all methods, the chromatin damage intensity and the percentage of sperm with fragmented DNA were determined.

RESULTS

While the DNA damage intensity and the % of sperm with fragmented DNA were significantly (P<0.05) correlated in direct methods (TUNEL, TUNEL with decondensation, alkaline and neutral) and CMA3, they were not associated in indirect methods. Percentages of SDF determined by alkaline Comet were significantly (P<0.05) correlated with TUNEL following decondensation, and CMA3; those determined by neutral Comet were correlated with the percentage of High DNA Stainability (SCSA); those determined by SCSA were correlated with neutral and alkaline SCD, and those determined by neutral SCD were correlated with alkaline SCD.



TAKE HOME MESSAGE

Percentages of sperm with fragmented DNA are directly related to the extent of chromatin damage when direct methods are applied in pig sperm, this is not the case of indirect techniques.

EFFECT OF GRADIENT VITRIFICATION SOLUTIONS AND TEMPERATURE ON SURVIVAL RATE OF CRYOPRESERVED BLACK SOLDIER FLY EMBRYOS

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FreezeM

Introduction

Cryopreserved embryos of Black Soldier Fly (BSF, *Hermetia illucens*) can be used for stock management and long-term preservation of genetic strains for the beneficial insect industry. Prior studies on embryos cryopreservation of several non-*Drosophila* flies concluded that published *Drosophila melanogaster* embryo cryopreservation protocols were not directly suitable for use with BSF embryos.

Materials and methods

In this work BSF embryos were dechorionated, permeabilized, loaded with cryoprotectant, dehydrated and cryopreserved. Level of viability was assessed by embryonic development and hatching. Stage 15-16 embryos, incubated at +29°C, were found to be at the most suitable stage for this treatment.

Results

The chemical solutions used for eggs permeabilization were composed of alcohol (isopropanol and butanol) and alkane (hexane, heptane) and Limonene:2-Methyltetrahydrofuran. It was shown that hatching was in the range of 35-55% after different protocol permeabilization (table 1). However, survival of single embryos after cryopreservation was found only after using L:Me at stage permeabilization (table 1.7)

Table 1. Hatching (% ± SE) of BSF eggs after application of different chemical solutions for permeabilization

N`	Alcohol	Alkane		Survival (%)
		Compound	Expose time (s)	
1	Butanol	Heptane + 0.3%butanol	60 s	57.1± 9.5
2	Butanol	Hexane + 0.3% butanol	40 s	28.7± 4.7
3	Butanol	Hexane + 0.5 %butanol	40 s	39.8± 6.6
4	Butanol	Hexane + 1.0 %butanol	40 s	61.5±10.3
5	Butanol	Hexane + 1.5% butanol	40 s	49.8± 8.2
6	Isopropanol	Heptane + 0.3% isopropanol	30 s	35.5± 5.9
7	Isopropanol	Limonene-2Me (7:1)	60 s	42.1 ± 7.1
8	Isopropanol	Limonene-2Me (7:1) => 5 sec in heptane	60 s+5 s	55.5 ± 9.3

Our peeling successfully remove the relevant layers

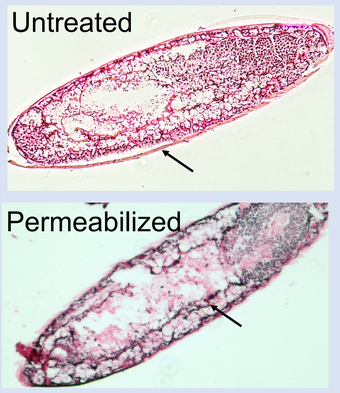


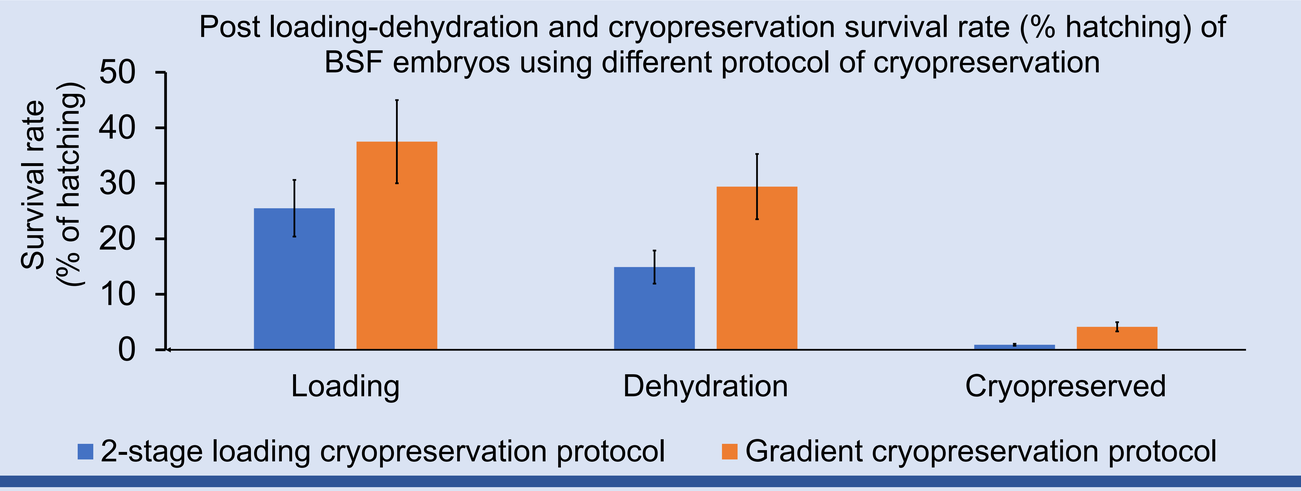
Fig 1 H&E staining of embryos without treatment compared to ones after the permeabilization protocol

Results (2)

We tested different protocols of cryopreservation (scheme 1). Protocol 1 was similar to cryopreservation protocol for *Drosophila*. It has been shown that a gradual loading up to 52% of the cryoprotectant concentration at +22°C has advantages in the survival of embryos comparing with 2-stage loading of embryos (at +30°C and +4°C). About 35-40% of embryos after gradient loading of vitrification solution hatched into larvae, compared with 10-15% when using 2-stage loading. We found that embryos performed best at equilibration time of 35 min. Vitrification solutions containing EG, FBS and trehalose (Tre) showed better survival rates compared to embryos treated with vitrification solutions containing EG, PEG and Trehalose.

Table 2. Cryopreservation protocol

	2-stage loading cryopreservation protocol	Gradient cryopreservation protocol
Loading EG	10% EG, 10 %FBS, 1% PSA in Schneider at 30 C, 30 min	10% and 20% EG in Schneider + 0.125M Tre, 5 % FBS, 1% PSA , RT, 10 min
Dehydration EG	44% EG, 0.5M Tre, 6% PEG 8000, 10% FBS, 1% PSA in Schneider on ice,15 min	40 % and 52 % EG solution in Schneider + 0.25M Tre, 10% FBS, 1% PSA, RT, 10 min
Freezing	plunged into liquid nitrogen	plunged into liquid nitrogen
Removing EG	0.5M Tre, RT, 2 min	0.75M Tre, 40% EG, 1 min➔0.5M Tre 20% EG 1min➔ 0.5M Tre, 1%PSA, 1 min => 0.25M Tre, 1% PSA, 1 min => 2 washes in Schneider, 10% FBS, 1% PSA for 5 min



Conclusions

- We establish a cryopreservation protocol, suitable for the Black soldier fly embryos
- Further optimization of the cryoprotectant loading, vitrification and thawing steps is required
- Pre-chilling treatment improved viability before freezing but did not improve viability after freezing

Vitrification of mouse cumulus-oocyte complexes selected with brilliant cresyl blue; an improved protocol for immature oocyte vitrification

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Introduction

Oocyte vitrification is the best option for maintaining fertility in girls and women who are at risk of infertility. Despite the advantages of immature oocyte vitrification compared with mature oocyte, the success rate of this technique are still unsatisfactory, and needs further improvements in *in vitro* maturation (IVM) and vitrification procedures. Previous studies have shown that the use of competent oocytes selected by BCB staining in the IVF and somatic cell nucleus transfer has improved the efficiency of these assisted reproductive techniques. On the other hand, cumulus cells regulate the processes of cytoplasmic and nuclear maturation of the oocytes, and fertilization. However, the cumulus cells are usually removed and denude oocytes enter the vitrification process. In the present study, maturation rate and developmental capability of mature and immature mouse vitrified oocytes with cumulus cells were evaluated.

Methods

Oocytes were collected from C57BL/6 female mice ovaries. After selection of competent immature oocytes with brilliant cresyl blue test, they were distributed in each group. COCs in fresh group didn't enter the vitrification-thawing procedure. Metaphase II (MII) oocytes were obtained after in vitro maturation (IVM) of BCB+ cumulus-oocyte complexes (COCs), and oocyte maturation was assessed. COCs and MII oocytes were vitrified utilizing a two step method at room temperature. They were loaded on a cryotop device (Kitazato, Japan), and immediately plunged into liquid nitrogen for one month storage. After warming process, COCs transferred to the IVM medium, and maturation rate was evaluated. Following the vitrification-warming procedure, survival rate and ROS levels in the IVM medium were examined. Also, the blastocyst developmental rate was assessed after parthenogenetic activation of oocytes using an inverted microscope.

Results

The ROS levels in vitrified COCs was lower than in vitrified MII group, but this difference was not statistically significant. Maturation rate and BDR were reduced in vitrified COCs group compared to the fresh group ($P < 0.05$). Vitrified-warmed COCs showed a higher survival rate, and ability to develop to blastocyst stage compared to the vitrified-warmed MII oocytes ($P < 0.05$) (table 1).

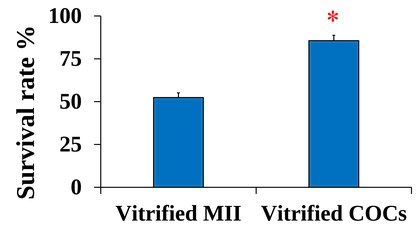
As shown in figure 1, survival rate of vitrified-warmed COCs significantly higher than that vitrified-warmed MII ($P < 0.05$). ROS levels were significantly higher in the vitrified groups than in the fresh group ($P < 0.05$).

Table 1. Embryo developmental rate of mouse competent oocytes with or without cumulus cells and ROS levels after vitrification-warming procedure.

Groups	Oocyte maturation (%)	BDR (%)	ROS (Relative light units/sec)
Fresh	80.7 ± 2.4	60.5 ± 4.2	46.8 ± 2.2
Vitrified MII	78.7 ± 3.1	21.4 ± 3.1[a]	92.8 ± 3.1[a]
Vitrified COCs	44.3 ± 1.7[a,b]	34.3 ± 3.7[a,b]	84.2 ± 3.7[a]

Values are as mean ± SEM. [a] $P < 0.05$ compared to fresh group; [b] $P < 0.05$ compared to vitrified MII group. MII, metaphase II oocyte; COC, cumulus oocyte complex. BDR, blastocyst development rate.

Figure 1. Survival rate of mouse competent oocytes with or without cumulus cells after vitrification-warming procedure. *$P < 0.05$



Discussion

Vitrification procedure reduces the developmental potential of the oocytes by severely damaging their structure and increasing ROS production. Optimization of current oocyte vitrification procedure is important to increase survival rate, fertilization and embryo development rate. It was demonstrated that vitrification of immature oocytes can be beneficial in establishing better cytoskeletal organization and developmental competence into the blastocyst compared to vitrification of MII oocytes. Our results indicated that vitrification of competent immature oocytes in presence of surrounding cumulus cells was more efficient than the vitrified MII oocyte and could ameliorate embryo developmental competence. More researches at the molecular level seems necessary.

ID: 33

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Equilibrium vitrification of mouse oocytes using lower concentrations of cryoprotectants

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Introduction

In **conventional vitrification**, mouse oocytes/embryos are vitrified in a non-equilibrium state. Therefore, they must be preserved in liquid nitrogen (LN_2). To enable handy transportation of vitrified embryos in dry ice, we developed a **near-equilibrium vitrification** method using a solution containing low concentrations of cryoprotectants, in which mouse embryos at multiple developmental stages were vitrified in a near-equilibrium state using EDFS10/10a. The vitrified mouse embryos can survive after being kept at -80°C for 4-28 days. In the present study, we examined whether this method is effective for mouse oocytes.

Methods

Vitrification solution: We used 10%(v/v) ethylene glycol (EG), 10%(v/v) DMSO, 24%(w/v) Ficoll and 0.4 M sucrose in PB1 (EDFS10/10a) for vitrification solution, and 5%(v/v) EG and 5%(v/v) DMSO in PB1 for pretreatment solution (PS).

Vitrification and warming: Mature oocytes were pretreated with PS at 25°C for 1-5 min, treated with EDFS10/10a at 25°C for 1 min, vitrified in LN_2 using a straw, and then kept at -80°C for 0-28 days. The straw was warmed in water at 25°C and CPAs in oocytes were removed in 0.5 M sucrose in PB1 for 5 min, 0.25 M sucrose in PB1 for 5 min, and then in PB1 at 25°C.

Assessment of viability of oocytes: ***In vitro*** survival of oocytes was evaluated by the appearance just after warming. Then, oocytes were submitted to zona cutting and fertilized with fresh sperm, and cultured to the blastocyst stage. ***In vivo*** survival was evaluated by developmental ability to term. Oocytes were vitrified, kept at -80°C for 4 days before being warmed, fertilized as described above, and transferred into recipient mice at the 2-cell stage.

Results

We examined the viability of oocytes pretreated with PS for 1-5 min, treated with EDFS10/10a, vitrified in LN_2, and stored at -80°C for 4 days before recovery. The survival rates after storage at -80°C for 4 days were high in all groups.

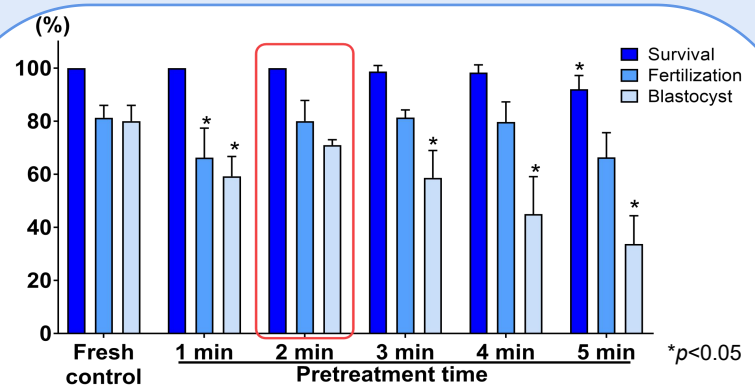

Fig 1.Optimum condition for equilibrim vitrification using EDFS10/10a

However, the rates of fertilization and blastocyst formation were high only in 2 min-pretreatment group. Therefore, 2 min-pretreatment protocol was adopted for subsequent experiments.

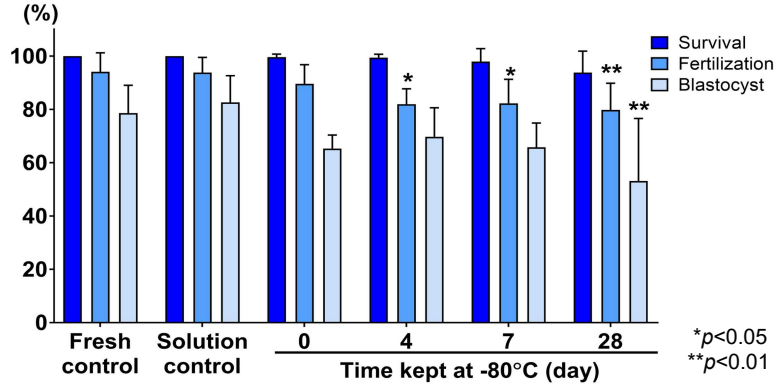

Fig 2. Viability of oocytes vitrified in LN_2 using EDFS10/10a, and then kept at -80°C for various periods before warming

To evaluate the extent of supercooling, vitrified oocytes were kept at -80°C for 4-28 days, a high proportion of oocytes survived and fertilized even after storage for 28 days (Fig. 2). However, the blastocyst formation slightly decreased as the keeping period at -80°C was extended.

Then, we compared the cell number in blastocysts derived from fresh oocytes and vitrified oocytes. There was no significant difference among fresh oocytes, vitrified oocytes, and oocytes vitrified and then kept at -80°C for 4 days (Fig. 3, left). However, there was an overall delay in blastocyst formation in vitrified oocytes (Fig. 3, right). These results suggest that vitrification reduce the viability of oocytes slightly.

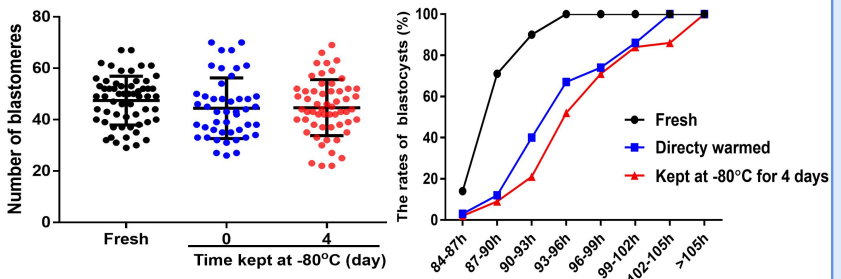

Fig 3. The number of blastomere in blastocysts (left) and the development speed to the blastocyst stage (right) derived from oocytes vitrified, kept at -80°C for 4 days, and *in vitro* fertilized

Finally, we confirmed developmental ability *in vivo* of vitrified oocytes. Oocytes were vitrified, kept at -80°C for 4 days, fertilized with fresh sperm, and transfered to recipient mice at the 2-cell stage. High proportions of the embryos were implanted and develop to term (Fig. 4). There were no significant differences in the rates between fresh and vitrified oocytes.

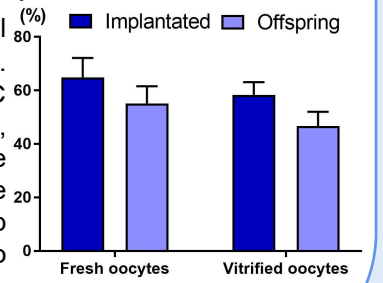

Fig 4. *In vivo* development

Conclusion

Mature mouse oocytes can be vitrified in a near-equilibrium state with the same method for mouse embryos. This method would enable convenient transportation of vitrified oocytes using dry ice.

Conflicts of interest: All Authors = none ***E-mail: keisuke@kochi-u.ac.jp**

Low-temperature phase transitions in dormant grape buds

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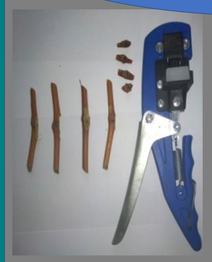
Introduction

The effective saturation of grape buds with cryoprotective media is complicated by heterogeneity of their structure, a large number of different channels and cavities with air in them. Traditional methods of saturation, based on the gradual diffusion distribution of fluid in the plant tissues, have low efficiency.

Purpose

to study the low-temperature phase transitions in grape buds during vacuum infiltration vitrification.

Materials and methods



Dormant grapes buds

Vine (Russian Concord) single-bud cuttings were collected in autumn and winter

Standard vitrification

VIV

Passive saturation

- incubation in PVS
- normal atmospheric pressure;
- saturation time in PVS - 60 minutes.

Active vacuum-infiltration saturation

- Degassing time: 5-15 minutes;
- Pressure: 20-40 kPa;
- Saturation time in PVS: 10-15 minutes.

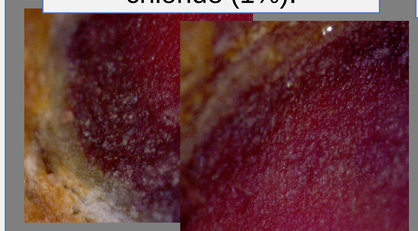
Snap freezing and storage in liquid nitrogen

Saturation efficiency

Low temperature differential scanning calorimetry (DSC) Thermograms of frozen samples (-196°C, 200°/min) were recorded while rewarming at 0.5 °/min.

Grape buds' integrity assessment

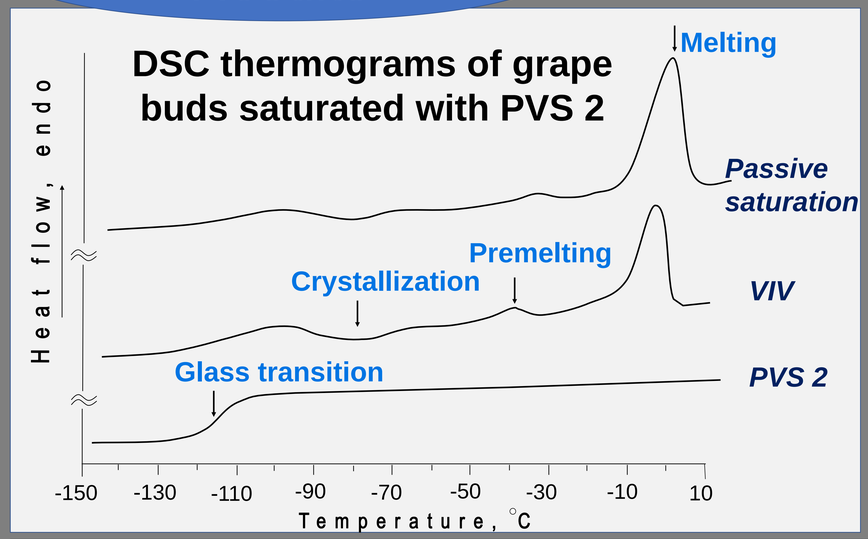
Staining with 2,3,5-triphenyltetrazolium chloride (1%).



Grape buds' viability assessment

Buds swelling during cultivation under phytotron conditions with 5500 lux lighting at a temperature of 18-20°C

Results



When saturated with PVSs, significant changes in heat absorption jump during glass transition, temperatures of phase transitions and their enthalpies were found. After saturation with PVS2, the temperature and enthalpy of melting were significantly lower than for PVS1, PVS3 and PVS4, which indicated a better saturation with PVS2. This may be due to the rather high content of Me_2SO.

Saturated buds	T_g, °C	T_c, °C	T_{pm}, °C	T_m, °C
Control	-87.9±1.6	-64.8±1.4	-37±0.5	-2.4±0.5
PVS1	-107.1±0.5*	-68.5±0.5	-37.2±0.5	-7.2±0.5*
PVS2	-115.1±0.5	-77.6±0.5	-37.1±0.5	-15.1±0.5*#
PVS3	-105±0.5*	-62.5±0.5	-36.4±0.5	-7.9±0.5*
PVS4	-107.2±0.5*	-77.4±0.5	-37.5±0.5	-9±0.5*
PVSN	-106.9±0.5	-85.2±0.5	-48.9±0.5	-4.1±0.5

Saturated buds	Δc_p	ΔH_c	ΔH_{pm}	ΔH_m
Control	9.1±1.0	0.83±0.21	0.38±0.16	174.1±29.8
PVS1	20.5±3.7*	9.22±1.15*	2.96±2.48*	68.3±9.9*
PVS2	18.0±0.9*	12.81±1.56*	4.5±0.51*#	55.72±6.34*#
PVS3	32.9±2.6*	1.98±0.56*	1.07±0.32*	116.7±17.3*
PVS4	34.8±3.9*	26.5±2.31*	0.42±0.13	73.6±11.5*
PVSN	12.3±2.1	1.33±0.17	0.79±0.13	123.5±11.5

* - statistically significant difference relative to control (p = 0.05), n = 5;
# –statistically significant difference relative to other PVSs (p = 0.05), n = 5 .

Conclusions

the vacuum infiltration method allows an effective saturation of grape buds with PVSs.

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Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine 2

Anna Mozgovska

Cryopreservation of apical and axillary sweet potato meristems by vitrification techniques

Researchers:

Anna Mozgovska, Natalia Bashtan, Tetiana Miroshnichenko, Tetiana Ivchenko 1

Nadiia Shevchenko, Olena Bobrova, Galyna Kovalenko 2

INTRODUCTION

Sweet potato (Ipomoea batatas) is the seventh most important food crop grown in many countries. Its root tubers are rich in sugars, which determine its taste, as well as in proteins, B vitamins, ascorbic acid, carotenoids, macro- and microelements. Sweet potato is vegetatively propagated, and a consistent supply of virus-free planting material is critical for sustainable production.

Cryotherapy is a promising procedure for eliminating pathogens from infected plant tissue.

The standard procedure usually involves cutting out the shoot tips, immersing them into liquid nitrogen and, after thawing and post-cultivation, regenerating into plants. For cryotherapy of plants, cooling conditions must allow survival of the cells only in apical dome and in the youngest (1st–2nd) leafs primordia. Shoot tips used for cryotherapy can be relatively large thus; they can show relatively high regenerative capacity. Cryotherapy has a number of advantages compared to traditional approaches, i.e. simplicity and time efficiency, ability to treat simultaneously a large number of samples ; low cost and high frequency of plants free from viruses after recovery.

The greatest challenge for the wider application of cryotherapy is that different genotypes of the same species may differently respond to cryotherapy.

In this work, we compared efficacy of different cryopreservation technique for the sweet potatoes meristems (Admiral variety) preservation.

RESULTS

It was shown that the regenerated rates of non-cooling meristems were 55% after 88% PVS3, 83% after PVS2 and 88% after modified PVS1 treatment. The post-thaw preservation rate was 68% after dehydration with PVS1, 38% with 88% PVS3 and 50% with PVS2 for cryopreservation in cryovials. After cryopreservation in aluminium pans the post-thaw survival rates was 82% for dehydration with modified PVS1, 46% with 88% PVS3, 73% with PVS2.

DSC thermograms of modified PVS 1, PVS 2 and 88% PVS 3 revealed only one heat capacity jump at temperature Tg (–109; –115.3; –93.9 respectively), associated with a reverse glass transition process. No exo- or endothermic peaks were recorded, indicating no crystallization, both at cooling and warming stage. This fact testifies that at the cooling stage the modified PVS 1, PVS 2, and 88% PVS 3 are completely transformed into a glassy state with highly stable amorphous phase, which does not crystallize even under slow warming above the glass transition temperature. The decrease in the number of viable meristems after exposure in 88% PVS 3 may be associated with the toxic effects of high concentrations of cryoprotectants or with osmotic reactions that lead to damage of specimens.

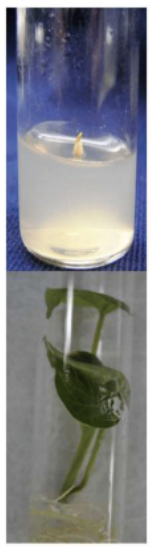


Fig. 3. Isolated meristem and plant of sweet potatoes

Fig. 4. Preserved meristems after cryopreservation in aluminium pans

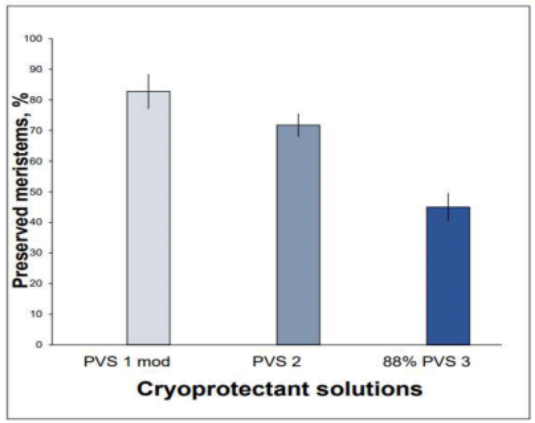
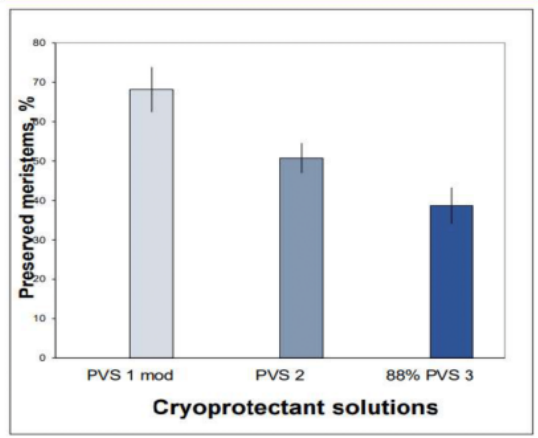


Fig. 5. Preserved meristems after cryopreservation in cryovials



MATERIALS AND METHODS

For cryopreservation the apical and axillary meristems of Admiral variety up to 1-2 mm were isolated from *in vitro* growth plants. They were dehydrated with plant vitrification solutions (PVS) and immersed into liquid nitrogen in 1.2 ml cryovials or 20 µl hermetic aluminium pans for differential scanning calorimeter (DSC).

The phase and glass transitions of PVSs in the temperature range from –196°C to complete media melting were investigated using low-temperature DSC. The thermograms were recorded during warming with the rate of 0.5 degrees/min.

Fig. 1. Hermetic aluminium pans, 20 µ

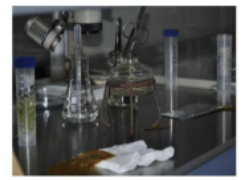
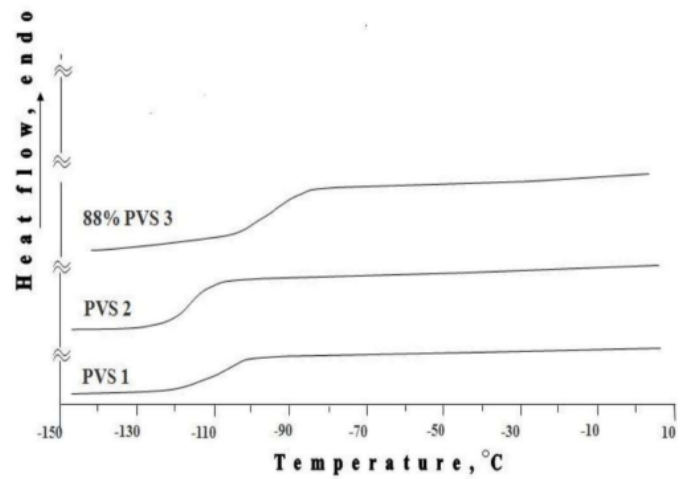


Fig. 2. Isolation meristems from shoot of sweet potato

Fig. 6. DSC termogram of different plant vitrification solutions



Cryo-light microscopy to study the freezing behavior of microalgae cells

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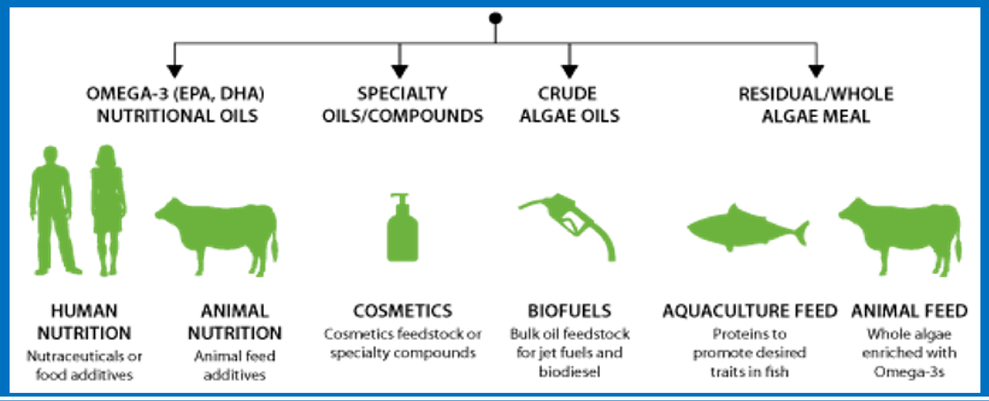
Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Kharkiv, Ukraine

introduction

Microalgae *Dunaliella salina* and *Chlorococcum dissectum*



The preservation of microalgae by regular reseeding on liquid nutrient media

✗ high risks of contamination;

✗ impossible in the face of large-scale pandemics, like a COVID-19.

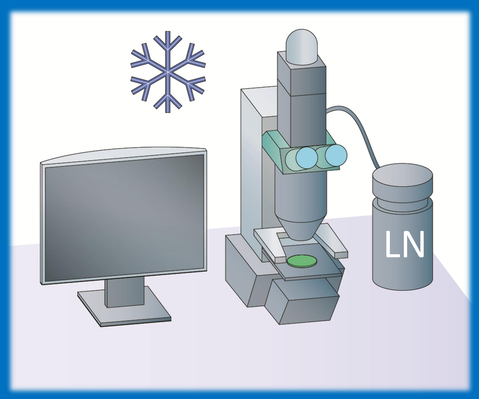
✓Cryopreservation is the most reliable way of long-term storage of plant objects with invariable genetic characteristics.

methods

To develop a cryopreservation protocol, it is important to understand what processes occur in cells at the freezing-warming stage.

We have made an attempt to establish a correlation between the processes of:

- dehydration-rehydration of cells;
- intracellular crystallization;
- cells preservation after freezing.



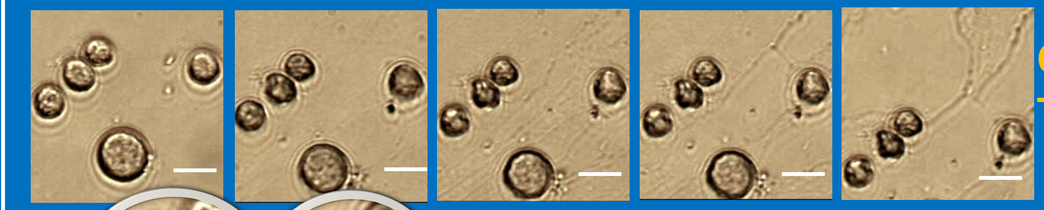
To visualize these processes, we assembled and used a cryo-light microscope.

Crystal formation at the cooling-warming stages depended on

- the type and concentration of cryoprotectants or their mixtures (PVS);
- the cooling rate;
- the composition of the cell culture medium.

results

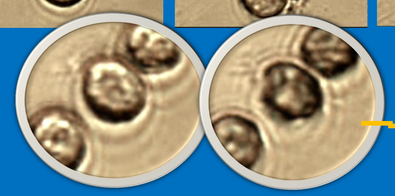
Chlorococcum dissectum (*cultivation on BG-11 medium*) cells:



Control

$T_{freez.}$ = –12°C

(the cooling rate is 6-8 degree/min)



cell dehydratation after extracellular crystallization

+20%Me_2SO

$T_{freez.}$ = –27°C

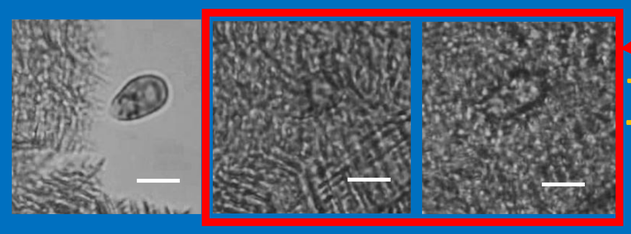
+ 15% EG

$T_{freez.}$ = –15°C

(the cooling rate is 30 degree/min)

Recrystallization

Dunaliella salina (*cultivation on Artari medium*) cells:



+ 50% PVS 2

$T_{freez.}$ = –75°C

(the cooling rate is 6-8 degree/min)

Cooling of the investigated cells of microalgae with different cooling rates with the addition of different cryoprotective media (scale bar = 20 μm)

The data obtained are a promising step towards the creation of effective protocols for cryopreservation of microalgae and other biological objects.

Investigations into glassy state of sugarcane shoot tips by PVS2 using Differential Scanning Calorimetry

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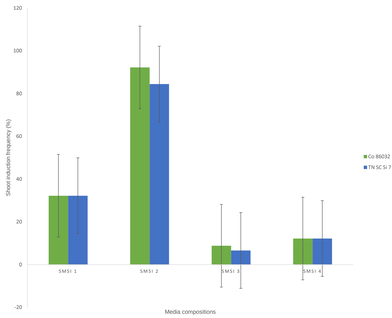
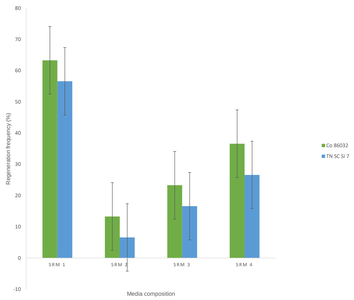
ICAR - Indian Agricultural Research Institute, New Delhi, India
Tamil Nadu Agricultural University, Tamil Nadu, India

Introduction

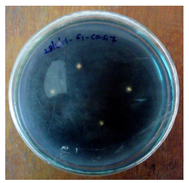
- *In vitro* regeneration and vitrification were optimized towards developing an efficient cryo-conservation.
- DSC was performed to study the thermal behavior of the both control and cryo-treated explants in order to ascertain cryo-fitness and to fix optimum vitrification treatments.
- Assessment of structural integrity of cryo-stored shoot apices using Scanning Electron Microscopy was done to identify the ultrastructural variations due to cryo-storage of shoot apices.



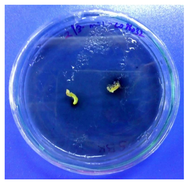
Effect of growth regulators on shoot induction & proliferation in the selected sugarcane genotypes



Effect of growth regulators on regeneration frequency in the selected sugarcane genotypes



Inoculated shoot apices

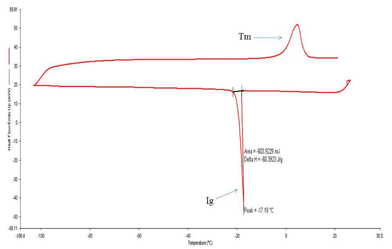


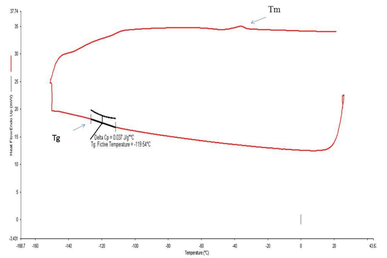
Shoot initiation after 20 days of inoculation

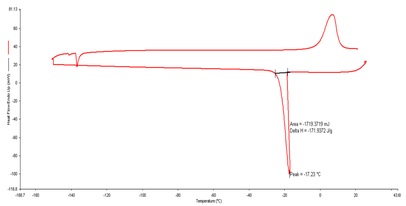
Shoot elongation

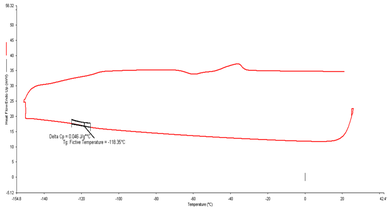
Proliferated Shoot

- Highest shoot induction frequencies of 92.2% and 84.4% were noticed in medium supplemented with 0.25 mg/l BAP + 5.0 mg/l kinetin + 0.05 mg/l NAA (SMSI 2) for Co 86032 and TN SC Si 7 genotypes
- Highest regeneration frequencies of 63.3% were in medium supplemented with 5 mg/l NAA (SRM 1) for Co 86032 and 56.6% was in medium supplemented with 5 mg/l NAA (SRM 1) for TN SC Si 7



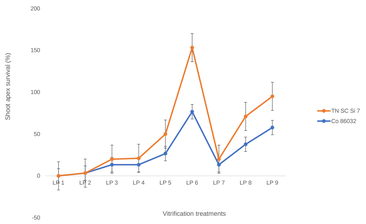




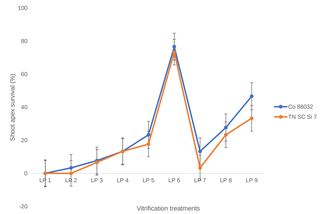


Thermogram curves for control shoot apices

Effect of vitrification on glass transition



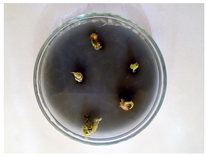
Effect of different vitrification treatment combination on survival of shoot apex of sugarcane genotypes after 24 h exposer in vapour phase of LN_2



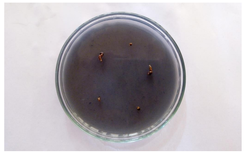
Effect of different vitrification factors in combination on survival of shoot apex of sugarcane genotypes after seven days of storage in vapour phase of LN_2

- Thermogram curves detected the formation of ice nucleation at a temperature of -17.1°C and -17.2°C in the control shoot apices of sugarcane genotypes.
- Melting in sugarcane control shoot apices showed ice melting at a temperature of 4.8°C and 4.5°C for both the genotypes.
- The DSC analysis conducted in Co 86032 shoot apices revealed that during cooling cycle, varying degrees of ice nucleation -49.2°C, -55.6°C, -43.9°C, -71.1°C, -68.9°C, -79.2°C, -80.5°C and -66.7°C was observed in treatments LP1, LP2, LP3, LP4, LP5 LP7, LP8 and LP9 respectively, as clearly correlated by variation in the downward peak pattern.
- Thermal characteristics data derived from DSC analysis shown that glass transition (Tg) was observed in LP6 at -121.7°C (Loading solution 20min and PVS-2 60min) treatments.
- LP6 treatments was observed to record very low enthalpy values of -0.04 J/g compared to that of control.

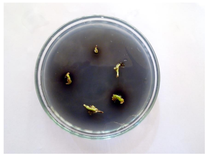
Effect of vitrification treatment on survival of shoot apex of sugarcane after exposure under vapour phase of LN_2



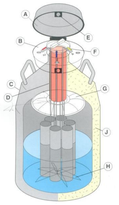
Shoot apices cultured on regeneration medium without LN_2 treatment (positive control)



Cryo treated shoot apices cultured on regeneration medium without PVS 2 treatment (negative control)

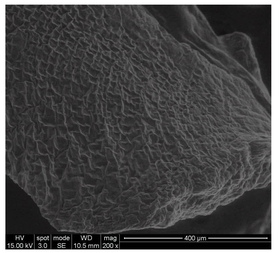


Cryo treated shoot apices cultured on regeneration medium after PVS 2 treatment (treated)

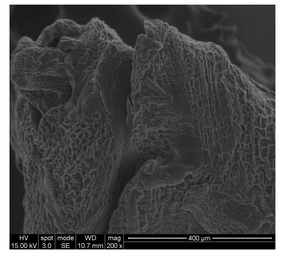


- Shoot apex survival percentage after cryo treatment was recorded 76.6%

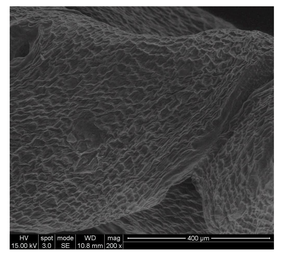
SEM image on cell structural integrity upon cryo storage of sugarcane shoot apices



Shoot apices cultured on pre cultured medium (positive control)



Shoot apices subjected to LN_2 treatment without vitrification (negative control)



Shoot apices exposed to LN_2 treatment after vitrification (treated)

- SEM analysis performed after LN_2 exposed shoot apices of sugarcane (Co 86032) revealed absence of distinct morphological variations at the cellular components following LS (20min) and PVS 2 (60min) treatment.
- The cryo-treated shoot apices cells were compact, distinct and intact.
- The cryo-treated shoot apices cells appeared as that of the normal untreated shoot apices.
- However, the negative control shoot apices of cassava (YTP 1) and sugarcane (Co 86032) showed major cellular disintegration and shrinkage of cell structures leading to the moderate to heavy cell damage after LN_2 exposure.

Conclusion

- A simple and reproducible vitrification based procedure for cryo-conserving sugarcane shoot apices was developed.

Shankar, M., Thiruvengadam, V., Shanmuganathan, M., Ganesh Ram, S. and Viswanathan, PL. (2018). Development of a simple and genotype independent in vitro regeneration system in Sugarcane [*Saccharum* spp] using shoot apex explants. Electronic Journal of Plant Breeding, 9(3), 1077-1082.

FREEZING DAMAGE ASSESSMENT IN EPIDERMAL TISSUE CRYOPRESERVED WITH ANTARCTIC YEAST ISOLATED TYPE1-ANTIFREEZE PEPTIDE

Muhammad Shuaib Khan[1,5], Adamu Abdul Abubakar[1],Sahar Muhammad Ibrahim[1], Mohd Basyaruddin Abdul Rahman[4], Mohd Zuki Abu Bakar[3] ,Noordin Mohamed Mustapha[2], and Loqman Mohammad Yusuf[1]*

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[4]Department of Chemistry, Faculty of Science, Universiti Putra Malaysia.
[5]Faculty of veterinary and animal sciences,Gomal university Dera ismail khan ,Pakistan
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Introduction

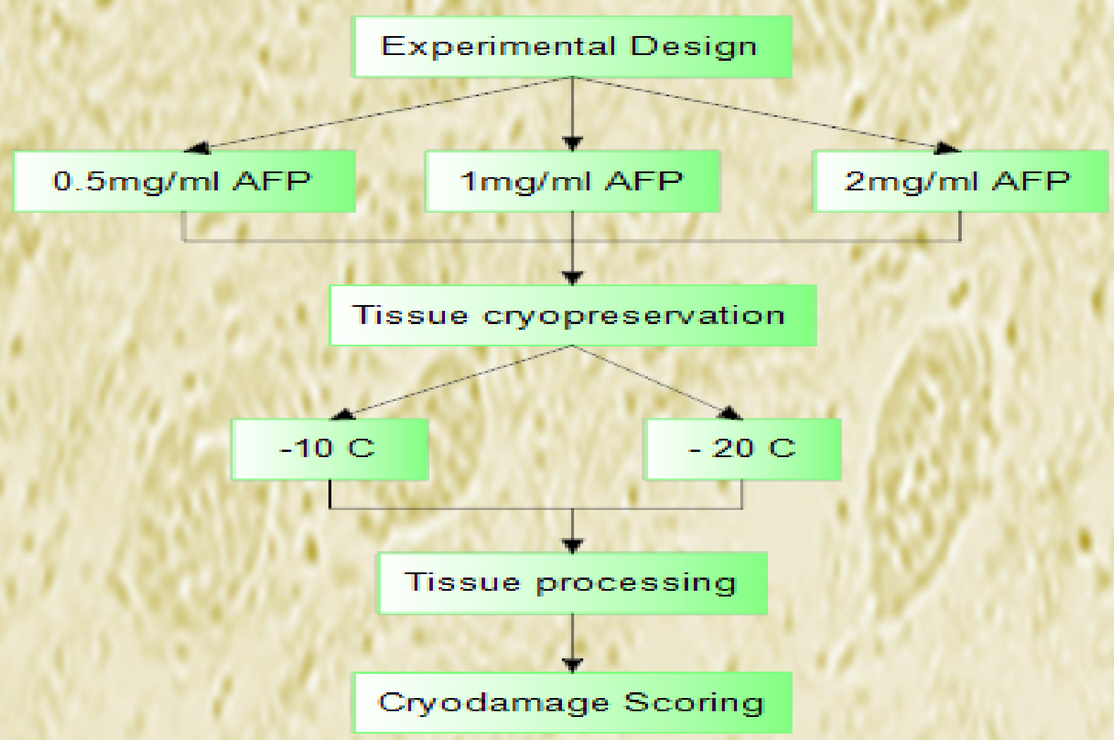
- Larger ice crystals increase the possibility of physical damage within frozen tissues (Griffith *et a*l., 1997). Overwintering plants and animals adopt two strategies namely freeze tolerance and freeze avoidance to survive at low and subzero temperatures. The extractions of antifreeze proteins or peptides (AFP) from different sources have been considerable research interest to improve tissue viability (Shah *et al*., 2012). AFP possess very important applications in the field of medicine, such as tissue and their membranes as well as organ preservation and cryosurgery (Amir *et al*., 2003)

Objective

- To evaluate freezing effects of cryopreservation of integumentary tissue in low AFP concentrations at different sub-zero temperatures using different microscopic techniques

Materials and Methods

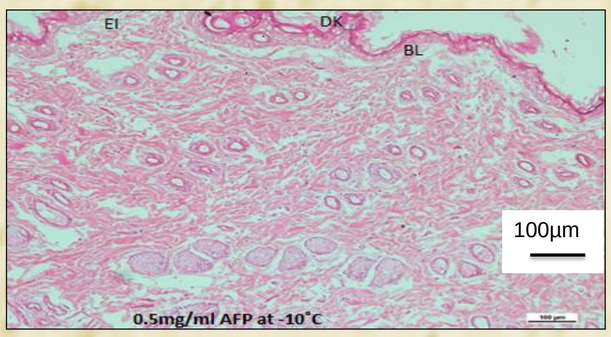
- Twelve (n=12) adult rats >30 day-old were used. Healthy skin tissues from abdominal area were harvested for the experiment. Tissue was processed and histological assessment was done according (Khaleel *et al*., 2014)

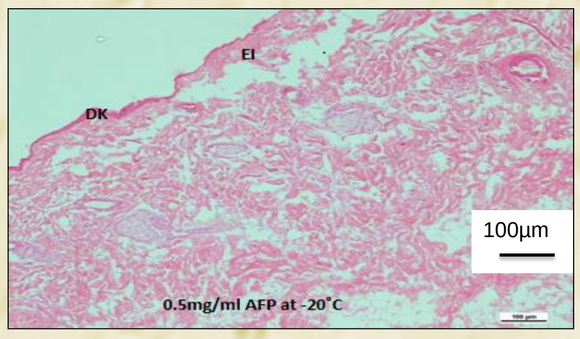


Results

- Histological studies showed that there was relatively consistent high damages at the epidermal region of the skin tissue cryopreserved at each 0.5mg/ml, 1mg/ml, 2mg/ml AFP solution for 24 hrs. at -20˚ C with high degree de-keratinization and tissue breakage (Fig. 1).
- There was moderate damage recorded in the epidermis of the skin tissue cryopreserved at 2 mg/ml AFP for 24 hrs. at -10 ˚ C (Fig.1 & Fig. 2)

Results





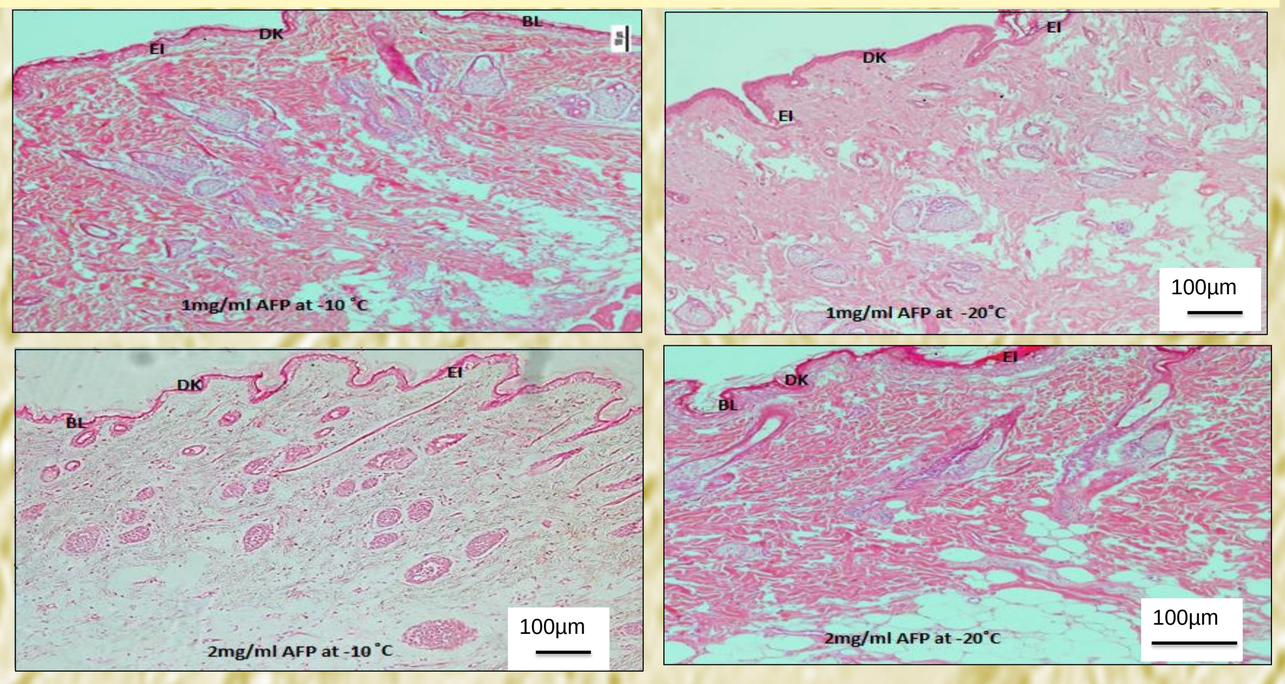


Figure 1: Micrographs of skin tissue cryopreserved in 0.5mg/ml, 1 mg/ml and 2mg/ml of AFP showing tissue damage at --20 ˚ C in Epidermal region of tissue denoted by de-keratinization (DK), epithelial integrity (EI), and ballooning of the epithelial cells (BL). Scale bar =100µm; x10 objectives.

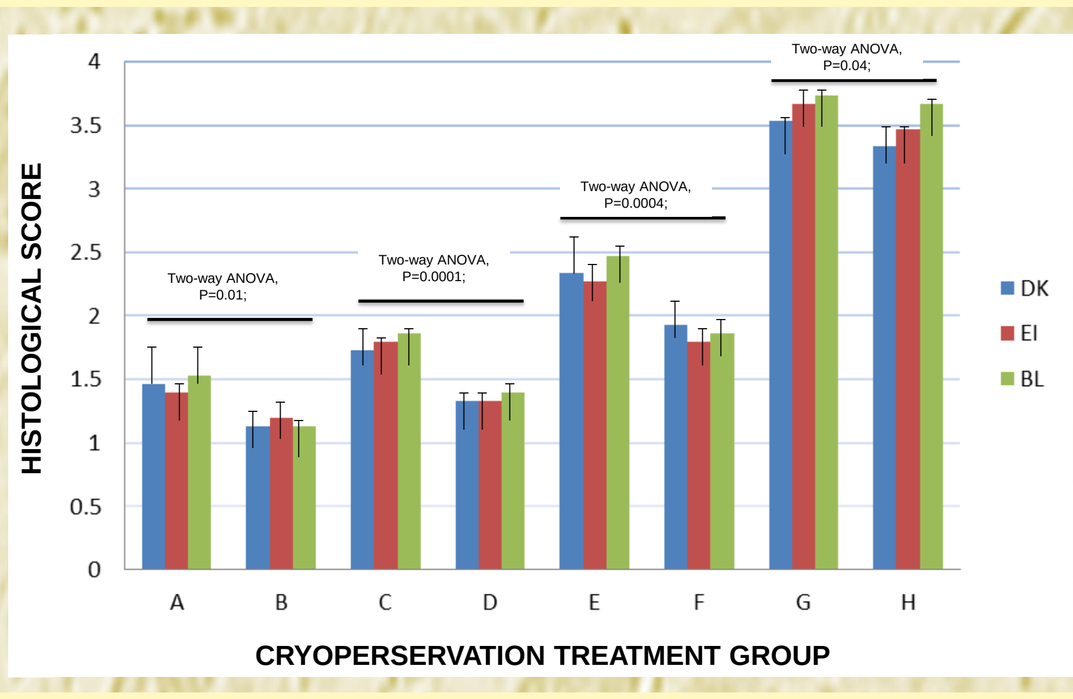


Figure 2: **Microscopic evaluation of skin tissue post-cryopreservation.** Histological assessment scores showed overall significant difference (P < 0.04; Two-way ANOVA) among different concentrations group, at -10 and -20˚C in epidermal region of skin tissue cryopreserved in 0.5, 1 and 2mg/ml AFP, along with control (80% glycerol), denoted by de-keratinization (DK), epithelial integrity (EI), and ballooning of the epithelial cells (EI). A-0.5mg/ml (-10˚C); B-0.5mg/ml (-20˚C); C-1.0mg/ml (-10˚C); D-1.0mg/ml (-20˚C); E-2.0mg/ml (-10˚C); F-2.0mg/ml (-20˚C); G-Control (-10˚C); H-Control (-20˚C);

Conclusion

- In summary our finding demonstrates that skin graft cryopreservation using low AFP concentration in relatively higher level, at - 10 ◦C gave better tissue preservation results than the tissues cryopreserved in lower concentration and at lower sub-zero temperature *i.e.* -20 ◦C. Future study will be looking into the use of relatively higher AFP concentration that able to cryopreserve living tissue for clinical tissue graft and transplantation to treat damaged and diseased tissues.

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The effect of cryoirrigation and cryopreserved placenta extract on the content of NO in the gastric mucosa in rats with diclofenac sodium-induced gastropathy

Fedir Hladkykh, Mykola Chyzh

Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine

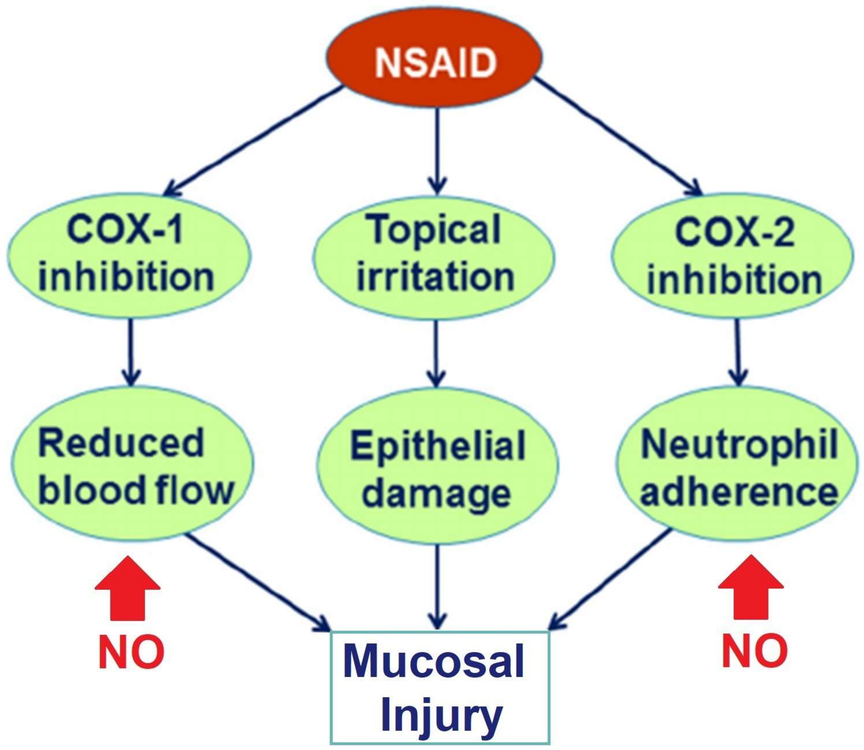
Finding new ways to decrease the ulcerogenic action of nonsteroidal anti-inflammatory drugs is an important task of modern cryomedicine and gastroenterology. It is known that nitrogen monoxide (NO) is a powerful vasodilating agent able to augment the blood supply to the mucous membranes. As a secondary mediator, NO is involved in the vasodilating effects of the vagus nerve and many other vasoactive substances.

Materials and methods. Acute diclofenac sodium-induced gastropathy in male rats was reproduced by a single intragastric administration of diclofenac sodium at a dose of 50 mg/kg. Euthanasia of animals was performed after 24 hours. Cryopreserved placenta extract was administered intramuscularly at a dose of 0.16 ml/kg body weight. The content of NO metabolites in the gastric mucosa was determined by spectrophotometric method, based on the oxidation of nicotinamide adenine dinucleotide phosphate during the reaction of NO formation with L-arginine and measured as light absorption at a wavelength (λ) of 340 nm.

Results of the research. The study showed that the introduction of diclofenac sodium led to a decrease by 41.5% ($p<0.05$) of NO metabolite levels in the gastric mucosa homogenates of rats relative to intact animals and amounted to 550 mmol/g of tissue. These findings were consistent with literature data on the ability of nonsteroidal anti-inflammatory drugs to form endogenous nitrogen monoxide resulting from inhibition of NO synthases.

Administration of cryopreserved placenta extract resulted in an attenuation of diclofenac-induced decrease in NO content in the gastric mucosa which amounted to 780 mmol/g of tissue, and was only 13.3% lower than that of the intact animals ($p<0.05$). Cryoirrigation of the gastric mucosa, similar to the introduction of cryopreserved placenta extract, lowered diclofenac sodium-induced decrease in NO metabolites. Combined cryoirrigation and administration of cryopreserved placenta extract led to a statistically significant ($p<0.05$) increase in NO level in gastric mucosa homogenates, completely eliminating diclofenac sodium-induced changes and amounted to 940 mmol/g of tissue, which was 1.1% higher than the respective value in the intact group of animals.

Conclusions. The obtained data indicate the ability of cryopreserved placenta extract, as well as the action of low temperatures to decrease the diclofenac sodium-induced NO reduction in the gastric mucosa. We suggest this being a mechanism of their gastrocytoprotective action.



by M. Magierowski, K. Magierowska, S. K.,T. Brzozowski Gaseous Mediators Nitric Oxide and Hydrogen Sulfide in the Mechanism of Gastrointestinal Integrity, Protection and Ulcer Healing. Molecules 2015, 20, 9099-9123; doi:10.3390/molecules20059099

DETECTION AND CHARACTERIZATION OF ANTIFREEZE ACTIVITY FROM BRASSICA JUNCEA LEAF CUTICLE

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***Corresponding author, [#]Presenting author**

PMPP Laboratory, DU

Introduction **Antifreeze proteins (AFPs)** possess the ability to restrict the growth of ice crystals. Earlier we purified dual functioning AFPs like **chitinases** (34, 3, 24, 10 kDa) and polygalacturonase inhibitor proteins (**PGIP**, 41 kDa) from laboratory grown seedlings of **Seabuckthorn,** a cold hardy shrub growing in the Himalayan cold deserts of India (Gupta and Deswal 2012; Sharma et al 2016). The efficacy of purified AFPs for cryopreservation of RBCs was tested. The preliminary results have suggested that AFPs enhanced RBCs survival by providing protection from hemolysis of cryopreserved rat RBCs suggesting its potential for cryopreservation of RBCs. Here, we attempted to explore the ***Brassica juncea*** **leaf cuticle,** the first line of defense for the presence of AFPs.

Methodology

- Cuticle protein sample was isolated from *Brassica juncea* leaves following organic extractant method (**Pyee et al., 1994).**
- 23 AFPs were present in the protein sample as revealed from nano LC-MS/MS data.
- Cuticle protein sample was then tested for the presence of antifreeze activity using nanoliter osmometer.

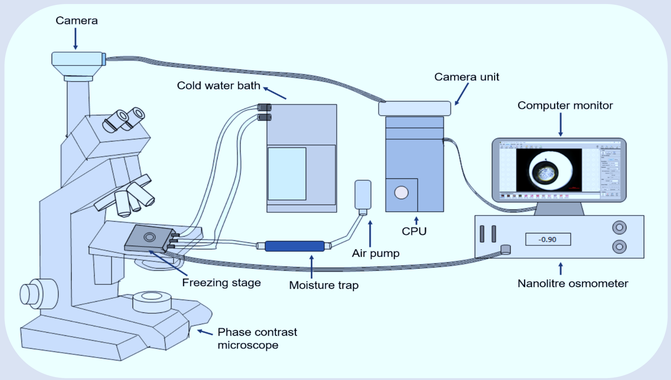
➢**Antifreeze activity assay (Instrument Setup)**

➢**Antifreeze molecules were proteins, saccharides or both?**

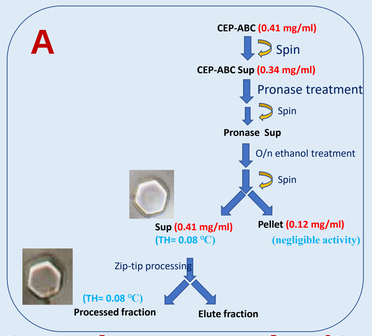
Pronase treatment → Protein degradation

Ethanol treatment → Saccharide separation

Zip Tip process → Peptide removal



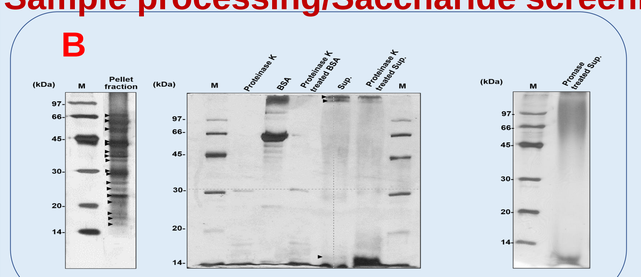
➢**Phenol sulphuric acid assay for saccharide content and FTIR, and NMR for characterization of antifreeze molecules**



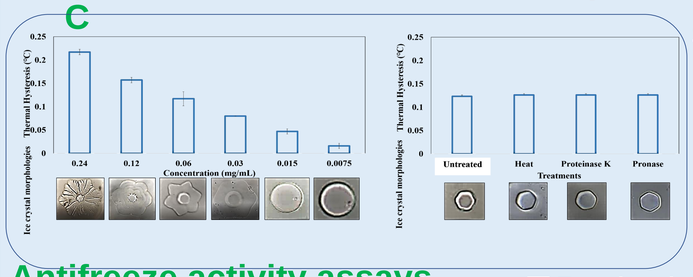
Results

- Both treated and untreated supernatants formed similar hexagons with same TH.
- **Peptide removal had no effect on thermal hysteresis** supporting that the antifreeze activity is due to saccharides only
- Complete degradation of polypeptides was achieved by **pronase** treatment

Sample processing/Saccharide screening

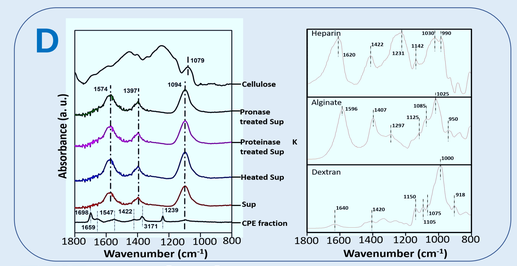


Gel profile of control & treated samples



Antifreeze activity assays

- Activity measured in the supernatant at 0.24 mg/ml of protein showed TH value above 0.2 °C which consistently decreased with dilutions. However,
- Heat and **protease-resistant antifreeze activity** with a thermal hysteresis value of 0.123 °C (at a protein concentration of 0.21 PPM) and hexagon-shaped ice crystal was observed



FTIR analysis

- Peaks in the supernatants at 1574 cm^{-1}, 1397 cm^{-1}, and 1094 cm^{-1} corresponded to peaks near 1600 cm^{-1}, 1400 cm^{-1}, and 1100 cm^{-1}in the common saccharides.
- FTIR analysis revealed the presence of **saccharides** in the supernatant

References **1. PMID: 22486727 (**DOI: 10.1021/pr200944z**) , 2. PMID: 8203911 (**DOI: 10.1006/abbi.1994.1263**), 3**. *Jour. Of Proteins & Proteomics 7(3), 2016, pp. 199-*

Acknowledgement **This study was supported by the Department of Biotechnology, India [DBT-IBSD project (IBSD/A1/P(PH-2))/4) and UGC, India].**

FIRST SPERM CRYOPRESERVATION PROTOCOLS FOR IBERIAN THREATENED FRESHWATER SPECIES: IBERIAN TOOTHCARP (*Aphanius iberus*) AND VALENCIA TOOTHCARP (*Valencia hispanica*)

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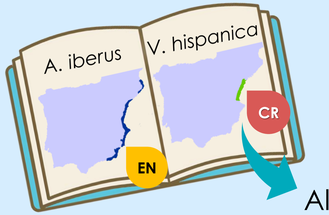
[2] Centro de Conservación de Especies Dulceacuícolas de la Comunitat Valenciana (CCEDCV), Valencia, Spain.

- INTRODUCTION -



Endangered species, autochthonous from the wetlands of the Iberian Peninsula

Albufera Natural Park (València, Spain)

Aphanius iberus

Valencia hispanica

Why are both endangered?
- Invasive species
- Loss of habitat
- Population reduction

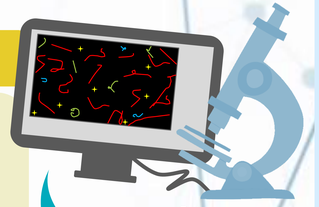
What measures are being taken?
- *Ex situ* conservation
- Reintroductions
- Habitat restoration

How could we help?
- Reproductive biology
- Gamete preservation
- Cryobanking

- MATERIAL AND METHODS -

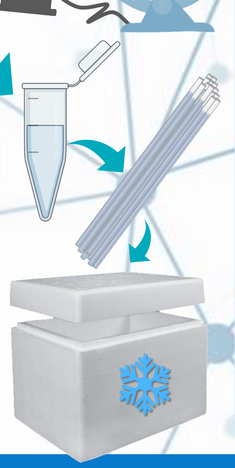
Sperm quality assessment

- Kinetic parameters (CASA-mot software)
- Morphometric parameters (ASMA software)



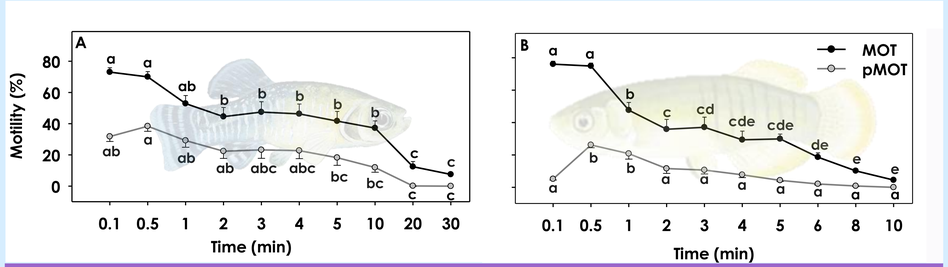
Cryopreservation protocol

- PBS medium
- Cryoprotector: 10% methanol
- Incubation: 15 min
- Liquid nitrogen ~ 2h
- Thawing: 40°C for 13s



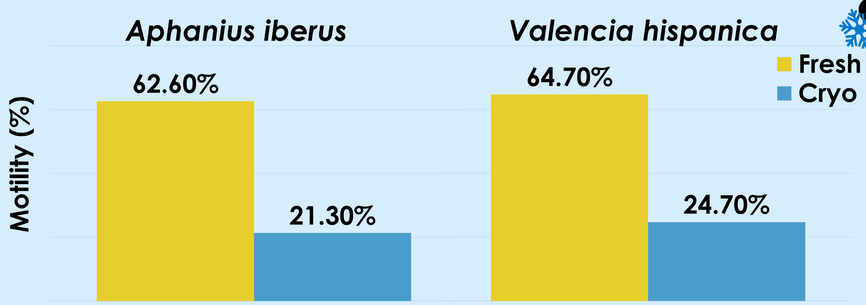
- RESULTS AND DISCUSSION -

- First sperm motility and morphometry evaluation for both species



	Area (μm²)	Perimeter (μm)	Length (μm)	Width (μm)
Aphanius iberus	4.84 ± 0.06	8.04 ± 0.05	2.55 ± 0.01	2.35 ± 0.01
Valencia hispanica	4.81 ± 0.04	8.02 ± 0.04	2.55 ± 0.01	2.32 ± 0.01

First sperm cryopreservation protocols for both species



First sperm quality study for both species

Cryopreservation as a method of conservation

For future work... ¿First cryolarvae?

Acknowledgments. This project has received funding from the Generalitat Valenciana (GV; Valencia, Spain) under the "*Subvenciones para la realización de proyectos de I+D+I desarrollados por grupos de investigación emergentes (GV/2019/130)*". VG has postdoc grant from the MICIU (IJCI-2017-34200) and MBG has PhD grant from the Universitat Politècnica de València (PAID-01-20), respectively.
Society for Cryobiology - CRYO2021

DEVELOPMENT OF A PROTOCOL FOR THE CRYOPRESERVATION OF PUFFERFISH (*Takifugu alboplumbeus*) SPERM

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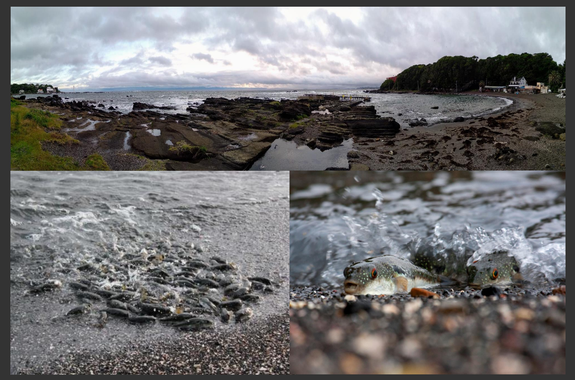
INTRODUCTION

The pufferfish *Takifugu alboplumbeus* lives in the Northwest Pacific Ocean and, like other related species (24 in the genus *Takifugu*), is considered a model organism, especially interesting for scientists due to its small genome. We set up a protocol to **cryopreserve the pufferfish sperm** and confirmed its usefulness using thawed sperm in an **IVF trial**.

MATERIAL & METHODS

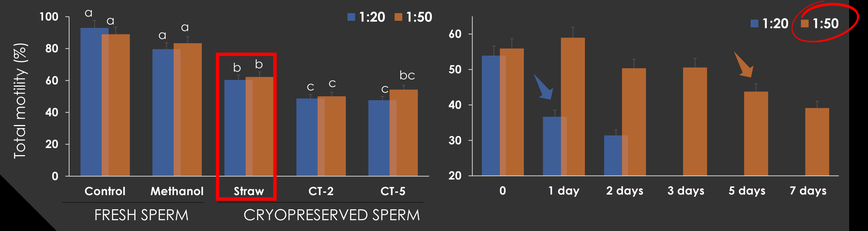
Sperm and eggs samples

They were obtained from fish caught during their natural spawning in **Arai Beach** (Miura, Japan) and later maintained in aquaria (18 °C) until stripping.



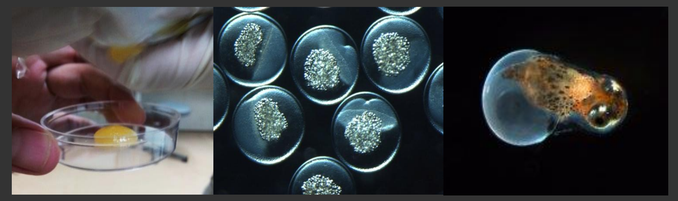
Cryopreservation trial

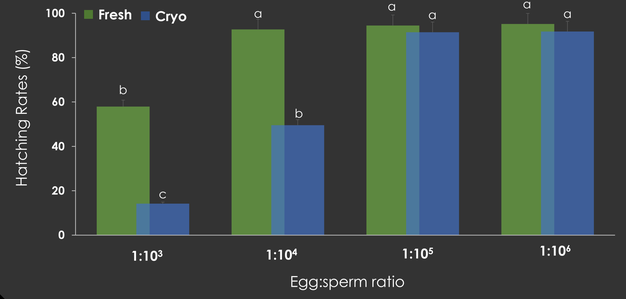
- Selection of sperm samples with high motility (>80%)
- Dilution of samples in SLS + 10% methanol
- Dilution ratios (1:20, 1:50)
- Vials (straws 0.5 ml, cryotubes 2 and 5 ml)
- Freezing & thawing conditions (Herranz-Jusdado et al., 2019)
- Sperm motility tested after thawing and after 1-7 days



In vitro fertilization trial

- Eggs from 1 female; sperm from 3 males
- Both fresh and cryopreserved sperm
- Different egg:sperm ratios (1:10^3, 1:10^4, 1:10^5 and 1:10^6)
- Fertilization rate (4-cells stage)
- Hatching rate





RESULTS

- Cryopreserved sperm samples frozen in **straws showed better results**, reaching post-thawed motilities >60% in both dilutions (1:20 and 1:50).
- Sperm samples from straws (diluted 1:50) were able to keep acceptable **motility values (around 40%) after 7 days**.
- **High (>90%) fertilization and hatching rates** were reached using 1:10^4 and 1:10^5 ratios (with fresh and cryopreserved sperm, respectively).
- The **egg:sperm ratio necessary** to achieve high hatching rates in pufferfish **is 10-times higher for cryopreserved than for fresh sperm**.

This study has laid the bases for the establishment of cryopreservation protocols in pufferfish, that will be helpful for further reproduction in captivity programs and genetic cryobanking.

References

Herranz-Jusdado et al., 2019. European eel sperm storage: Optimization of short-term protocols and cryopreservation of large volumes. Aquaculture 506 (2019) 42–50.

Acknowledgements

Generalitat Valenciana funded the stay in Japan of JFA, LPI and VG (BEST 2018/093, /112, /124). VG has a postdoc grant from the MICIU (Juan de la Cierva-Incorporación; IJCI-2017-34200).

CRYO2021 VIRTUAL CONFERENCE | JULY 20-23

EFFECT OF LOW TEMPERATURE STORAGE IN SEA URCHIN EGGS VIABILITY

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INTRODUCTION

Cryopreservation of sea urchin eggs is yet to be successfully achieved as is well known that the cell is easily damaged by the exposure to CPAs and the freezing process. The information about sea urchins chilling-injury is scarce.

AIM

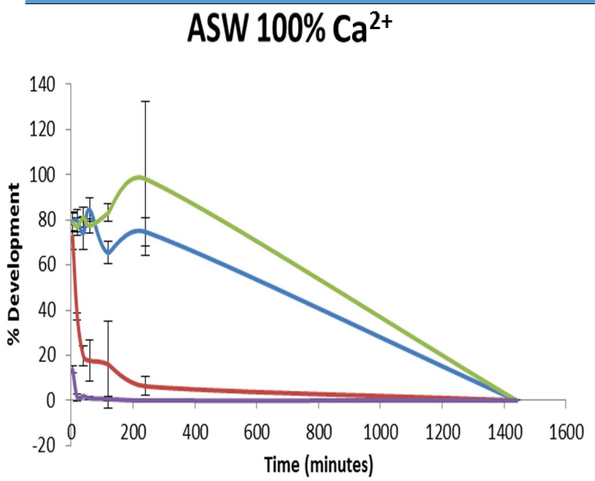
Test the possibility of extending sea urchin eggs viability without freezing them.

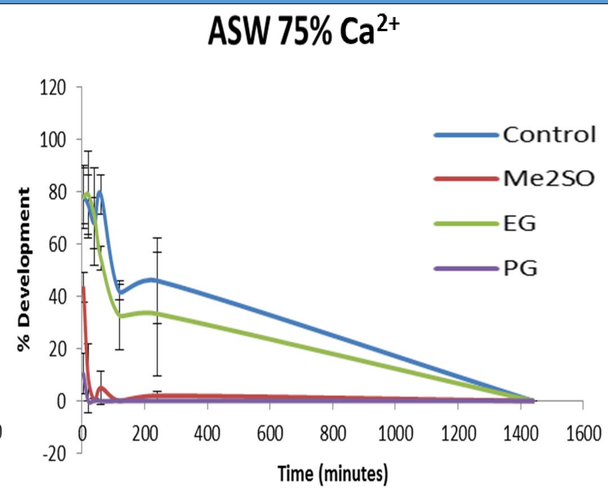
METHODS

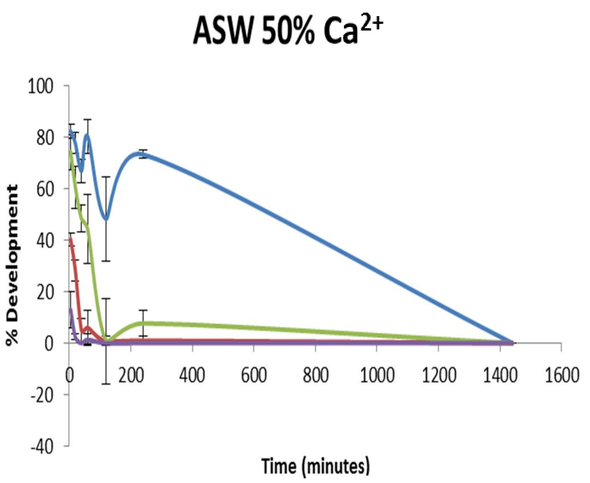
Eggs were kept at 4ºC for 5', 20', 40', 1h, 2h, 4h and 24h using artificial sea water (ASW) with 100%, 75%, 50%, 25% and 0% of Ca^{2+}. Also, 1M of Dimethyl sulfoxide (Me_2SO), Ethylene glycol (EG) and Propylene glycol (PG) was added. Then they were transfer to fresh sea water, fertilized and incubated for 48h.

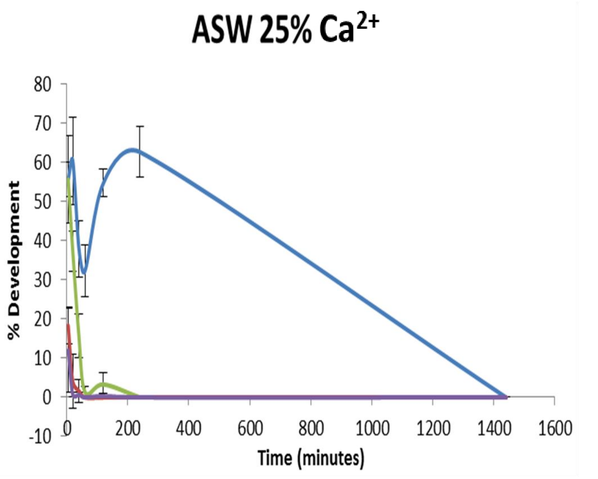
Fertilized egg and female gonads.

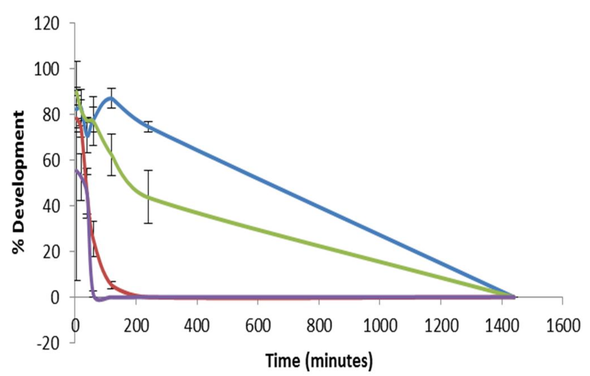
RESULTS











Percentage of development through time for ASW with decreasing [Ca^{2+}] and different CPA.

- Best CPA → EG (80% development)
- Worst CPA → PG (15% development)
- Development drops from 85% at 5' until there is no development at all after 1 day.
- [Ca^{2+}] → slightly decreased in development.

CONCLUSION

Using ASW with lower concentrations of Ca^{2+} do not protect against **chilling injury**, neither does the presence of CPAs without affecting their viability. Sea urchin eggs can be kept at room temperature (18ºC - 20ºC) for 8h and still be perfectly viable.

ACNOKLEGMENTS

I'd like to thank Estefanía Paredes and Jesús Troncoso from the University of Vigo (Spain), and Dominic Olver and James Benson form the University of Saskatchewan (Canada) for the brainstorming.

Effects of penetrating cryoprotectants on sperm cryopreservation of Pacific abalone, *Haliotis discus hannai*

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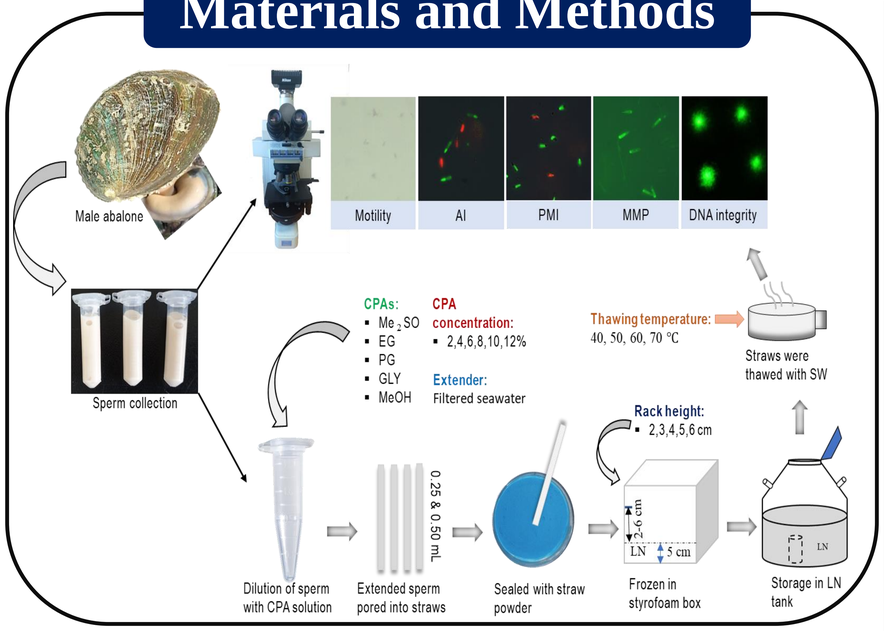
Abstract

Pacific abalone, *Haliotis discus hannai* is a high commercial seafood in South-East-Asia. This study aimed to improve a sperm cryopreservation protocol for Pacific abalone using Dimethyl sulfoxide (Me_2SO), ethylene glycol (EG), propylene glycol (PG), glycerol (GLY), and methanol (MeOH) as cryoprotectants (CPAs). Four different equilibration time (5, 10, 30, and 60 min), and two types of equilibration temperature (4℃ and 20℃) with different concentration of CPAs (2, 4, 6, 8, 10, and 12%) were selected at the present experiment. Most equilibration temperatures with each CPA showed significant differences among different equilibration time. Post-thaw sperm motility of five CPAs showed no significant difference at two equilibration temperature. Based on these results, 8% Me2SO, 8% EG, 6% PG, 2% glycerol, and 2% methanol were chosen to determine optimal conditions for sperm cryopreservation. The highest post-thaw sperm motility (8% Me_2SO : 50.6%, 8% EG: 45.6%, 6% PG: 28.7%, 2% GLY: 44.5%, 2% MeOH: 25.4%) was achieved after exposing sperm to liquid nitrogen (LN2) vapor for 10 min at 5 cm above the LN2 surface and then submerging them in LN_2 for at least 2 h followed by thawing at 60℃ with seawater. In this study, 8% Me2SO, 8% EG, 6% PG, 2% GLY, and 2% MeOH were chosen to check post-thaw sperm quality to estimate percentages of plasma membrane integrity (PMI), mitochondrial potential analysis (MP), and acrosome integrity (AI) using fluorescent techniques. Sperm cryopreserved using 8% Me_2SO revealed significantly higher PMI (54.2 ± 2.0%), MP (42.8 ± 2.1%), and AI (50.5 ± 2.3%) than those cryopreserved with 8% EG, 6% PG, 2% GLY or 2% MeOH. The current study has demonstrated that 8% Me_2SO was optimal for sperm cryopreservation for *H. discus hannai* with 5 min of equilibration time, 5 cm of rack height and 60℃ of thawing temperature.

Materials and Methods



Note: Me_2SO: Dimethyl sulfoxide; EG: ethylene glycol; PG: propylene glycol; GLY: glycerol; MeOH: methanol

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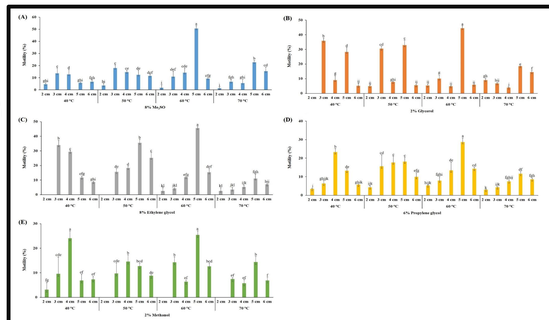

Fig. 1. Effects of different rack heights and thawing temperatures with five CPA combinations on post-thaw sperm motility.

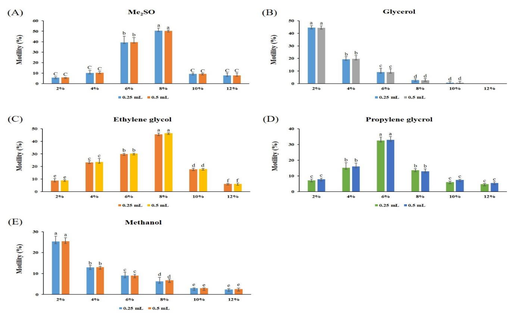

Fig. 2. Effect of different container types (0.25 mL and 0.5 mL straws) with five CPA combinations on the percentage of post thaw sperm motility.

Results and Conclusion

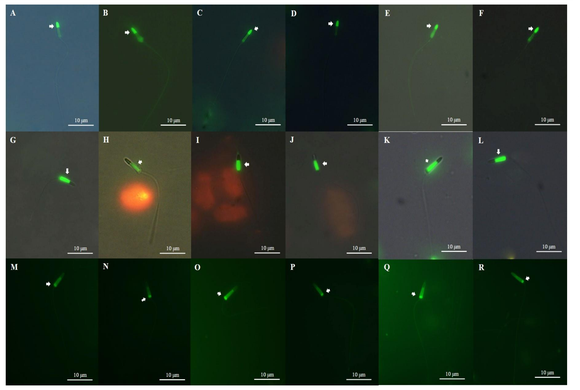

Fig. 3. Fluorescently stained photographs of Pacific abalone sperm. **(A)** Acrosome integrity (AI) of fresh sperm; **(B)** AI of sperm cryopreserved with 8% Me_2SO; **(C)** AI of sperm cryopreserved with 8% ethylene glycol (EG); **(D)** AI of sperm cryopreserved with 6% propylene glycol (PG); **(E)** AI of sperm cryopreserved with 2% glycerol (GLY); **(F)** AI of sperm cryopreserved with 2% MeOH; **(G)** Plasma membrane integrity (PMI) of fresh sperm; **(H)** PMI of sperm cryopreserved with 8% Me_2SO; (I) PMI of sperm cryopreserved with 8% EG; **(J)** PMI of sperm cryopreserved with 6% PG; **(K)** PMI of sperm cryopreserved with 2% GLY; **(L)** PMI of sperm cryopreserved with 2% MeOH; **(M)** Mitochondrial membrane potential (MMP) of fresh sperm; **(N)** MMP of sperm cryopreserved with 8% Me_2SO; **(O)** MMP of sperm cryopreserved with 8% EG; **(P)** MMP of sperm cryopreserved with 6% PG; **(Q)** MMP of sperm cryopreserved with 2% GLY; **(R)** MMP of sperm cryopreserved with 2% MeOH.

Table 1. Effect of different equilibration time for five CPAs on the percentage of sperm motility.

Concentration	Equilibration time	Me_2SO	Glycerol	Ethylene glycol	Propylene glycol	Methanol
2%	5 min	90.5 ± 0.8^{a}	67.7 ± 3.8^{a}	88.7 ± 1.2^{a}	89.0 ± 0.9^{a}	78.6 ± 1.0^{a}
	10 min	87.7 ± 1.0^{b}	66.9 ± 4.6^{a}	87.6 ± 0.6^{a}	85.7 ± 0.4^{a}	72.3 ± 1.5^{b}
	20 min	81.8 ± 1.2^{c}	65.6 ± 4.2^{a}	81.1 ± 1.5^{b}	78.3 ± 1.8^{b}	68.0 ± 1.5^{c}
	30 min	78.7 ± 1.5^{d}	60.6 ± 1.9^{a}	80.1 ± 2.0^{b}	73.7 ± 1.8^{c}	60.7 ± 2.8^{d}
	60 min	69.1 ± 0.8^{e}	43.3 ± 2.9^{b}	68.1 ± 3.0^{c}	62.8 ± 3.9^{d}	87.3 ± 1.9^{a}
4%	5 min	87.2 ± 1.3^{a}	60.3 ± 3.6^{a}	84.9 ± 1.0^{a}	83.7 ± 1.2^{a}	75.4 ± 1.2^{a}
	10 min	80.9 ± 1.0^{b}	50.2 ± 1.9^{b}	80.7 ± 1.6^{ab}	77.0 ± 1.3^{b}	70.4 ± 0.9^{b}
	20 min	79.3 ± 2.2^{b}	47.2 ± 1.3^{b}	77.6 ± 3.2^{bc}	71.3 ± 0.9^{c}	64.4 ± 1.9^{c}
	30 min	74.2 ± 1.4^{c}	40.1 ± 4.1^{c}	75.6 ± 2.1^{c}	67.4 ± 1.2^{d}	49.2 ± 1.4^{d}
	60 min	64.5 ± 0.6^{d}	18.9 ± 0.5^{d}	60.5 ± 3.8^{d}	47.7 ± 3.3^{e}	82.5 ± 1.0^{a}
6%	5 min	83.7 ± 0.9^{a}	52.9 ± 3.1^{a}	82.6 ± 0.5^{a}	78.7 ± 1.9^{a}	69.0 ± 1.5^{a}
	10 min	78.3 ± 0.1^{b}	46.8 ± 1.6^{b}	78.6 ± 1.9^{b}	73.0 ± 1.2^{b}	65.1 ± 2.0^{b}
	20 min	76.2 ± 0.9^{b}	39.5 ± 4.4^{c}	77.0 ± 1.1^{bc}	69.9 ± 0.7^{c}	54.8 ± 1.0^{c}
	30 min	72.1 ± 1.8^{c}	34.3 ± 1.3^{d}	75.4 ± 0.6^{c}	66.4 ± 1.1^{d}	47.4 ± 0.9^{d}
	60 min	58.5 ± 1.9^{d}	11.0 ± 1.4^{e}	47.2 ± 2.2^{d}	41.1 ± 1.4^{e}	78.9 ± 2.3^{a}
8%	5 min	78.2 ± 3.7^{a}	43.2 ± 4.2^{a}	77.9 ± 0.9^{a}	65.2 ± 3.1^{a}	57.9 ± 2.2^{a}
	10 min	75.3 ± 5.0^{a}	22.2 ± 3.9^{b}	75.8 ± 1.3^{a}	64.1 ± 1.1^{a}	51.9 ± 1.5^{b}
	20 min	72.6 ± 2.0^{ab}	19.2 ± 1.4^{bc}	72.9 ± 0.6^{b}	58.7 ± 0.7^{b}	49.6 ± 1.3^{c}
	30 min	69.4 ± 1.0^{b}	16.2 ± 2.1^{c}	70.4 ± 0.8^{c}	56.6 ± 0.8^{b}	39.2 ± 0.7^{c}
	60 min	45.1 ± 0.6^{c}	6.3 ± 1.7^{d}	43.0 ± 1.5^{d}	37.4 ± 3.0^{c}	77.8 ± 1.5^{d}
10%	5 min	68.4 ± 0.1^{a}	22.1 ± 4.0^{a}	70.3 ± 1.9^{a}	70.7 ± 1.9^{a}	54.5 ± 1.2^{a}
	10 min	60.4 ± 2.5^{b}	9.1 ± 1.7^{b}	66.6 ± 0.7^{b}	60.3 ± 1.8^{b}	51.5 ± 1.8^{b}
	20 min	51.8 ± 1.4^{c}	4.8 ± 0.3^{c}	58.6 ± 2.2^{c}	59.4 ± 2.2^{b}	46.2 ± 1.3^{b}
	30 min	49.8 ± 1.1^{c}	2.7 ± 0.4^{c}	46.6 ± 1.2^{d}	53.9 ± 2.3^{c}	34.5 ± 0.9^{c}
	60 min	37.4 ± 1.7^{d}	1.2 ± 1.0^{c}	34.0 ± 1.9^{e}	37.3 ± 1.4^{d}	72.2 ± 3.5^{d}
12%	5 min	58.3 ± 1.8^{a}	19.9 ± 1.0^{a}	63.6 ± 3.2^{a}	69.1 ± 1.4^{a}	52.7 ± 2.1^{a}
	10 min	53.3 ± 0.9^{b}	6.9 ± 1.4^{b}	55.9 ± 2.6^{b}	51.4 ± 3.4^{b}	48.3 ± 1.0^{b}
	20 min	46.5 ± 0.4^{c}	3.3 ± 2.0^{c}	46.0 ± 1.4^{c}	48.1 ± 1.5^{b}	45.2 ± 2.0^{bc}
	30 min	44.3 ± 1.7^{c}	1.9 ± 0.2^{cd}	45.4 ± 1.5^{c}	36.7 ± 0.4^{c}	25.1 ± 1.0^{c}
	60 min	24.2 ± 3.6^{d}	0.0 ± 0.0	30.3 ± 0.8^{d}	31.5 ± 0.9^{d}	78.6 ± 5.2^{d}

Table 2. Comparison of plasma membrane integrity (PMI), mitochondrial potential (MP), and acrosome integrity (AI) between fresh and cryopreserved sperm (mean ± SD) .

Cryoprotectant solution	PMI (%)	MP (%)	AI (%)
Fresh sperm	96.0 ± 0.7^{a}	94.1 ± 1.6^{a}	95.8 ± 0.7^{a}
8% Me_2SO	54.2 ± 2.0^{b}	42.8 ± 2.1^{b}	50.5 ± 2.3^{b}
8% EG	50.5 ± 4.0^{c}	39.4 ± 1.6^{c}	48.0 ± 2.2^{bc}
6% PG	31.2 ± 1.7^{d}	24.4 ± 0.9^{d}	28.7 ± 2.1^{d}
2% GLY	48.9 ± 1.8^{c}	37.9 ± 1.2^{c}	46.3 ± 2.9^{c}
2% MeOH	28.1 ± 2.7^{d}	20.8 ± 1.5^{e}	27.4 ± 2.5^{d}