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Cryobanking of Skin Cells from the Critically Endangered European Mink (*Mustela lutreola*)

[1]Trinidad Leon-Quinto, [2]Sisco Manas and [3]Arturo Serna
[1]Area of Zoology and Cell Bioengineering, Institute of Bioengineering,
University Miguel Hernandez, Av. Universidad s/n, 03202 Elche, Spain
[2]Biodiversity and Animal Protection Service, Departament of Agriculture,
Livestock, Fishing, Food and Environment, Catalonia Government-Generalitat de Catalunya,
Dr. Roux,80, 08017 Barcelona, Spain
[3]Area of Applied Physics, Department of Physics and Computer Architecture,
University Miguel Hernandez, Av. Universidad s/n, 03202 Elche, Spain

Abstract: Cryobanked somatic cells may be used when required for wide-ranging applications such as advanced veterinary reproductive biotechnologies, genetic resources conservation or studies at genetic level, among others. In populations where gametes are difficult to obtain such as threatened species, such cells acquire special relevance because they could be later reintroduced into the breeding pool by inducing pluripotency and/or nuclear transfer. Since, knowledge of specific cell response to cryopreservation process is required, the physiological sensitivity to cryoinjury of skin cells from the critically endangered European mink was evaluated in this research and for the first time. After thawing, the cell viability and functionality was investigated by analyzing their membrane integrity, metabolic activity, glycosaminoglycan production and proliferative capability. Freezing media included the permeating cryoprotectant dimethyl sulfoxide either at 5 or 10%. These two concentrations were investigated alone or along with the non-permeating cryoprotectant sucrose at 0.1 or 0.2 M, testing six different freezing solutions. Dimethylsulfoxide at 10% resulted in thawed cells exhibiting proliferative capabilities similar to those of fresh-unfrozen cells with mean values around 80, 90 and 85% for viability, metabolic activity and GAG synthesis, respectively. Dimethylsulfoxide alone at 5% resulted in a significantly decreased protection. However, the combination of 5%-dimethyl sulfoxide and sucrose (both 0.1 as 0.2 M) yielded similar results to those obtained with 10%-dimethylsulfoxide. The findings are relevant to preserve alive the genetic patrimony of the European mink as well as to provide properly cryobanked cells for a wide variety of applications. The results could be also useful for other mammals.

Key words: Skin, cryopreservation, mammal, threatened species, genetic resource bank, biodiversity conservation

INTRODUCTION

Advances in animal cell biotechnology are now a days increasing the interest on the creation of cell banks. Such reserves hold different cell types in order to be used when they are needed. Banking of cells is an important and useful approach for human applications (Erdag *et al*., 2002; Hirt-Burri *et al*., 2008; Lermen *et al*., 2010) as well as for threatened and domestic animals (Leon-Quinto *et al*., 2009a, 2011; Arat *et al*., 2011). Human health requires of research for progression of biomedicine by means of cell therapy, tissue engineering or regenerative medicine among others. Cell banking is also very relevant both for threatened and domestic animals and research conducted with frozen-thawed cells is innumerable. Results obtained from such studies are used in reproduction, toxicology, phylogenetic studies, conservation management, evolution or comparative biology among others.

Additionally, recent advances in biotechnology are showing the utility of using somatic cells to obtain induced pluripotency. Induced Pluripotent Stem (iPS) cells derived from fibroblasts is a new approach to potentially obtain gametes from somatic cells, since iPS cells could be later differentiated onto the required cell type (Takahashi and Yamanaka, 2006). In addition, iPS cells provide a source of reprogrammed donor cells for nuclear transfer. The generation of stable and characterized iPS cells to be used as donor nuclei may help to improve the

Corresponding Author: Trinidad Leon-Quinto, Institute of Bioengineering, University Miguel Hernandez, Av. Universidad s/n, 03202 Elche, Spain

efficiency of Interspecies Somatic Cell Nuclear Transfer (ISCNT) variant of SCNT used for endangered species. In fact, there is evidence that less differentiated cell types can increase SCNT efficiencies as compared to terminally differentiated cell types because they are more easily reprogrammed (Saito *et al*., 2003, 2004). Therefore, cryobanking somatic cells acquire a relevant role when gametes are difficult to obtain as in threatened species.

In the context of conservation biology, biomaterials including somatic cells are being preserved for Southern Africa's wildlife (Bartels and Kotze, 2006) the Frozen Zoo of San Diego (Ryder *et al*., 2000; Ryder, 2005) Hungary's domestic species and wildlife (Rzepiel *et al*., 2012, 2013) or the Frozen Ark Consortium (http://www.frozenark.org) among others. In the same way, the group created in 2003 a Biological Resource Bank for Spain's endangered wildlife including mammals, fishes and many avian species (Leon-Quinto *et al*., 2009b, 2011).

Despite efforts undertaken to conserve the endangered European mink, its global population is classified as critically endangered according to the IUCN (2013) red list (http://www.iucnredlist.org). Additionally, it is considered one of the most endangered mammals in Europe (Manas *et al*., 2003; Maran *et al*., 2011). During the 20th century, the number of European mink declined and its global distribution became reduced to three fragmented and separated populations (Maran *et al*., 2011). Genetic studies have shown that the Western populations (Spain and France) have very low genetic variability, the southern population slightly higher and the eastern populations have the greatest one (Michaux *et al*., 2005). In the family Mustelidae, studies related to embryos and semen cryobanking have been carried out (Amstislavsky *et al*., 2008). However, collection of gametes from threatened individuals is often difficult, especially from females (Leon-Quinto *et al*., 2011). Consequently, the banking of a genetic pool representative of the population biodiversity is very difficult when only gametes are considered. Somatic tissues and cells can be instead easily and harmlessly collected through biopsies from the male and female populations (Leon-Quinto *et al*., 2011). Cryobanking of somatic tissues and cells allows therefore the preservation of the largest genetic biodiversity, a relevant aspect in threatened species.

One of the most widely used cryoprotectants in skin banking is dimethyl sulfoxide (Me_2SO) (Erdag *et al*., 2002; Moritz and Labbe, 2008; Benkeddache *et al*., 2012), usually utilized alone or along with a non-permeating cryoprotectant. Me_2SO has been extensively used as a permeating cryoprotectant because of its high membrane permeability. However, despite the protection that the cryoprotectant offers to the cells during freezing and thawing, a risk of cell damage exists, especially when it is used in high concentrations (Fahy, 1986). Previous studies in different cell types and tissues have demonstrated beneficial cryoprotective effects of sugars as trehalose, sucrose or D-allose (Sui *et al*., 2007). In cryopreservation protocols, these sugars have been used as non-permeating cryoprotectants because of the negligible permeability of plasma membranes to their molecules thus stabilizing cellular membranes during freezing and possibly dehydrating cells so that excessive swelling and osmotic shock are avoided (Crowe *et al*., 1988). Me_2SO and sucrose have also been used together in several species, like Goldfish (Moritz and Labbe, 2008) for cells and tissues, human dermal fibroblasts and human engineered-dermal tissues (Wang *et al*., 2007), lamb cartilage (Cetinkaya and Arat, 2011) or Iberian lynx skin cells (Leon-Quinto *et al*., 2011).

To assure a proper cryopreservation process, species specific freezing conditions have to be developed (Woods *et al*., 2004). To the knowledge, the physiological sensitivity to cryopreservation process of somatic cells has never been evaluated neither in the European mink nor in any other mustelid. Taking into account the potential relevance of such cells and within a larger conservation program, the global goal was to establish optimal freezing procedures related to skin cells from the critically endangered European mink. For this purpose, researchers analyzed the impact of six freezing media on physiological properties of thawed cells as the cell viability (by analyzing their membrane integrity) and the cell functionality (by measuring their metabolic activity, glycosaminoglycan production and proliferative capability).

MATERIALS AND METHODS

Skin sampling: Researchers implemented protocols to collect and send samples and provided sampling kits with the appropriate media. The samples were either personally transported or sent by urgent courier in refrigerated Styrofoam containers. The time elapsed from the moment the samples were taken to its processing in the laboratory was under 24 h. Samples were taken by veterinarians from the breeding captivity program and consisted of millimeter-sized skin biopsies which were removed from the external surface of the ear and isolated always profiting from animal manipulation for other reasons (sanitary check-ups, etc.).

Samples were taken from twelve healthy 3 months old European mink born in captivity in El Pont de Suert Breeding Centrum (Lleida, Spain) in the framework of the Spanish captive breeding program.

Reagents, washing, culture and freezing media (reagents): Components for washing, culture and freezing media were purchased from Gibco/BRL (Grand Island, NY,USA) with the exception of MTT and GAG assays which were from Sigma Chemical Co. (Madrid, Spain) and Foetal Bovine Serum (FBS) from Biochrom (AG).

Washing medium: The washing medium consisted of D-MEM (Dulbecco's Modified Eagle's medium) supplemented with 25 mM Hepes, 100 U mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin and 1% fungizone. Such a medium was also used as transport medium.

Culture medium: Researchers established cell cultures following (Leon-Quinto *et al*., 2011). Briefly, the basal culture medium was composed of D-MEM (Dulbecco's Modified Eagle's medium) 25 mM Hepes, 1% non-essential amino acids, 0.1 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin. Such a basal culture medium was supplemented with FBS at 15%, glucose at 4.5 g L^{-1} and EGF at 10 ng mL^{-1}.

Freezing medium: Basic Freezing Medium (bFM) was composed of D-MEM supplemented with 25 mM Hepes, 1% non-essential amino acids, 0.1 mM 2-mercaptoethanol and FBS at 35% (v/v). Such a basic freezing medium was supplemented with cryoprotectants at several concentrations. Researchers selected two concentrations for the permeating dimethyl sulfoxide (Me_2SO) 5 and 10% v/v. Each Me_2SO concentration was investigated alone and along with the non-permeating cryoprotectant sucrose at 0.1 or 0.2 M, testing thus six different freezing solutions.

Cells cryopreserved in the combination sucrose/Me_2SO were first loaded with Me_2SO by incubation in bFM containing the appropriate concentration (5 or 10%) at 4°C for 15 min. Such samples were then transferred to the cryopreservation solutions containing both cryoprotectants Me_2SO (at either 5 or 10%) along with sucrose (at either 0.1 or 0.2 M) in bFM for an additional 15 min of incubation at 4°C. The cryovials containing 1 mL of each cryopreservation solution cooled at a cooling rate of 1°C min^{-1} until reaching -70°C and subsequently plunged in liquid nitrogen.

Cellular viability: After thawing, the viability of cells was evaluated by analyzing their membrane integrity by using the Trypan Blue Staining Method. The Survival Rate (SR) was calculated as the ratio of the number of cells presenting intact membranes after thawing to those cryopreserved x100. Viable cells were counted on a haemocytometer.

Cellular functionality: Cell functionality was evaluated in thawed cells by measuring their proliferative activity, metabolic activity and glycosaminoglycan content, through population doubling, MTT and Glycosaminoglycan assays, respectively. The mean values found were compared to those from the fresh control group.

Population doubling assay: The proliferative activity was evaluated by means of the Population Doubling (PD) rate which was calculated through the population doubling assay as follows: viable thawed cells were counted on a haemocytometer using the trypan blue exclusion assay (day 0) seeded and allowed to proliferate for 10 days, trypsinized at such a day and counted. The PD rate was calculated at day 10 by using the following equation:

$$\text{PD at day 10} = \log_2 \frac{\text{Cell number at day 10}}{\text{Cell number at day 0}}$$

MTT assay: Metabolic activity of thawed cells was measured via mitochondrial dehydrogenases, by means of the colorimetric 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. Mitochondrial dehydrogenases of metabolically active cells cleave the tetrazolium ring, yielding purple formazan crystals which are insoluble in aqueous solutions. The crystals formed were dissolved in acidified isopropanol and the resulting purple solution was spectrophotometrically determined from the absorbance values.

Briefly, thawed cells were seeded in triplicate at a density of 5×10^4cells mL^{-1} and cultured in a humidified atmosphere of 5% CO_2 at 37°C. After 10 days, 20 μL MTT solution (5 mg mL^{-1} in PBS) were added and the plates were incubated for an additional 4 h at 37°C. To achieve solubilisation of formazan crystals formed in viable cells, MTT solubilisation solution containing acidified isopropanol (200 μL) was added and the absorbance was then measured at a wavelength of 570 nm. For comparison, the fresh-control group was assayed and the mean value obtained was normalized to 100%.

Glycosaminoglycan (GAG) assay: GAG content of the cells was measured by spectrophotometric determination with Dimethylmethylene Blue (DMMB). Briefly, thawed cells were seeded in triplicate at a density of 2×10^4 cells mL^{-1}. After 10 days, the culture medium was drained and the cells were digested at 65°C for 4 h in papain extraction reagent (200 μg mL^{-1} papain, 5 mM L-cystein HCl, 0.1 M sodium acetate, 10 mM Na_2HPO_4, 5 mM EDTA, Sigma-Aldrich). Digested solution was mixed with DMMB working solution (1:1) and the

absorbance was immediately measured at a wavelength of 595 nm. The correlation between GAG concentrations and absorbance was calculated according to the standard curve (Sigma-Aldrich). The fresh-control group was assayed and the mean value obtained was normalized to 100% in order to be used as the reference for the cryopreserved cells.

Overall procedure: Skin samples taken from the external surface of the ear were washed several times in the washing medium, cut into similar millimeter sizes and seeded as in a previous research (Leon-Quinto *et al*., 2011). Cells around explants were allowed to proliferate for 10 days, trypsinized at such a day, counted and either seeded or frozen. One half was seeded on new gelatine-coated dishes and used to calculate the functionality of control fresh cells as specifically described above. For that, their proliferative activity was measured after 10 days of culture as well as their metabolic activity and glycosaminoglycan content.

The other half was frozen in the different freezing solutions commented above. After at least 4 weeks of cryopreservation, cells were removed from liquid nitrogen. Cryovials were then quickly thawed by plunging them in a water bath at 37°C (3-4 min) until the ice melted. In order to remove the cryoprotectants, thawed cells cryopreserved by means of Me_2SO were rinsed in bFM twice for 15 min. Cell samples preserved in sucrose/Me_2SO were rinsed in bFM containing either 0.1 or 0.2 M sucrose for 15 min at 4°C and then in bFM alone for another 15 min.

Once cells were thawed, cellular viability and functionality were evaluated as commented earlier and compared to the fresh control values.

Statistical analysis: Statistical analysis was performed by means of multi-factor ANOVA with second-order interactions included and single-factor ANOVA tests. Student's t-tests, Bonferroni and HSD Tukey tests were applied for multiple comparisons when convenient. Data are presented as mean±standard deviation. In order to detect statistical differences, a $p<0.05$ level of significance was applied in all the analyses. Each experiment was repeated at least three times.

RESULTS

Sensitivity to cryopreservation in cells from the European mink: To evaluate the ability of European mink skin cells to be cryopreserved we analysed their viability and functionality after thawing.

Cellular viability after thawing: The survival rate was used to evaluate the viability of thawed cells. As shown in Fig. 1, the survival rate parameter was significantly lower in cells cryopreserved only by means of 5% Me_2SO than in those from the rest of cryopreservation media ($p<0.05$% in all cases). Concerning cells preserved in the media containing 10% Me_2SO, researchers did not find any statistically significant difference among their survival rates after thawing, independently of the sucrose concentration, being in all cases around 80%. Therefore, the addition of sucrose to the freezing media containing Me_2SO at 10% did not affect the survival rate of thawed cells. Regarding thawed cells previously preserved in 5% Me_2SO, the survival rate fell to 52±4% in absence of sucrose. However, the addition of sucrose at both 0.1 and 0.2 M to the freezing media containing 5% Me_2SO, significantly increased their survival rates to 78±6 and 79±5%, respectively ($p<0.05$ for both cases). In addition, such values were similar to those obtained after cryopreservation in 10% Me_2SO.

Cellular functionality after thawing: Functionality of thawed cells was evaluated by means of several physiological properties as the proliferative and metabolic activities as well as the glycosaminoglycan synthesis.

Proliferative activity: Proliferative capacities of thawed cells were measured 10 days after thawing. The obtained values were compared to the fresh control as well as among them. As shown in Table 1, thawed cells from the three freezing media containing Me_2SO at 10% presented at day 10 after thawing similar PD rates when compared among them as well as to the fresh control, independently of the sucrose concentration used. Data from thawed cells

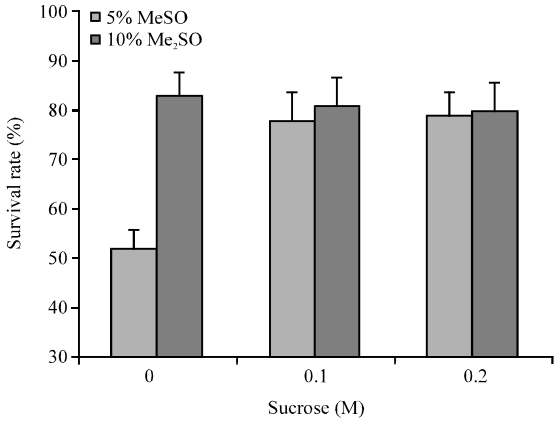

Fig. 1: Viability of cells after thawing, estimated by means of the survival rate. Cells were previously cryopreserved in freezing media containing Me_2SO at either 5 or 10% v/v. Each Me_2SO concentration was investigated alone and along with sucrose at 0.1 or 0.2 M. Data are percentage±SEM

Table 1: Proliferative activity of thawed cells previously cryopreserved in freezing media containing Me_2SO at 5 or 10% v/v either alone or combined along with sucrose at 0.1 or 0.2 M

Me_2SO (%)	Sucrose (M)	PD rate (day 10)
5	0	3.52±0.38[a]
	0.1	4.63±0.29[b]
	0.2	4.75±0.31[b]
10	0	4.83±0.44[b]
	0.1	4.76±0.36[b]
	0.2	4.69±0.35[b]
Fresh control	Fresh control	5.02±0.31[b]

Proliferative activity of cells was estimated by means of the Population Doubling (PD) rate at day 10 after thawing. Data are mean±SEM. The mean control value refers to that obtained for fresh unfrozen cells. Values with different superscripts (a, b) are considered significantly ($p<0.05$) different

from the freezing media containing 5% Me_2SO revealed different responses depending on the absence or presence of sucrose as additional cryoprotectant. Thus, cells cryopreserved by using only 5% Me_2SO presented a PD rate significantly decreased when compared to the fresh control cells as well as to those from the rest of the preservation media ($p<0.05$ for all cases). Nevertheless, the addition of sucrose at either 0.1 as 0.2 M to the freezing media containing Me_2SO at 5% significantly increased the proliferative capacity of thawed cells until similar values to those from fresh cells (Table 1).

Metabolic activity: Metabolic activity in thawed cells was estimated 10 days after thawing by means of the MTT assay. The findings were given in percentage respect to the fresh control group which was normalized to 100%. As Fig. 2 shows, thawed cells from the three freezing media including Me_2SO at 10% did not present significant differences among their metabolic activities, independently of the presence or absence of sucrose. In fact, such thawed cells exhibited metabolic activities of 91±5, 90±6 and 89±4 in presence of 0.0, 0.1 or 0.2 M sucrose, respectively. Concerning thawed cells cryopreserved in Me_2SO at 5% when sucrose (at both 0.1 and 0.2 M) was additionally added to the freezing media, cells exhibited similar mean values than those obtained for cells cryopreserved in 10% Me_2SO (Fig. 2). However, in the absence of sucrose in the freezing media, thawed cells exhibited a metabolic activity of 61±5% respect to the fresh control. Such a value was significantly decreased when compared to those from the rest of thawed cells ($p<0.05$ for all cases).

Glycosaminoglycan content: Extracellular matrix production from thawed cells was estimated at day 10 after thawing. Results from glycosaminoglycan content were in the same way than those from metabolic activity and proliferative capacity. Indeed, only cells cryopreserved by means of Me_2SO at 5% without sucrose, exhibited after thawing a significantly reduced

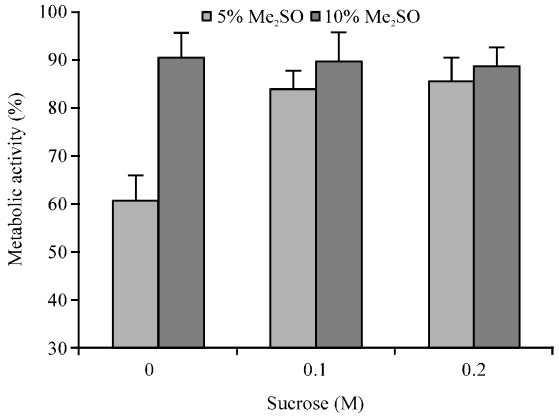

Fig. 2: Metabolic activity of thawed cells, evaluated by means of the MTT assay at day 10 after thawing. Results were represented in percentage compared to the fresh unfrozen control normalized to 100%

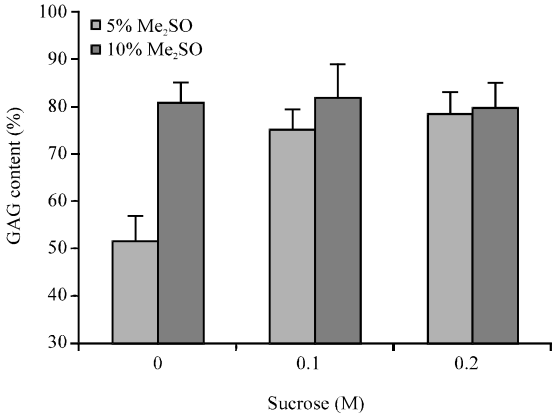

Fig. 3: Glycosaminoglycan (GAG) content of thawed cells, evaluated by means of the glycosaminoglycan assay at day 10 after thawing. Results were represented in percentage compared to the fresh unfrozen control normalized to 100%

extracellular matrix production of 59±5%. The rest of cryopreservation conditions yielded to glycosaminoglycan contents quite high in thawed cells when compared to the fresh control, being around 80% for all of them (Fig. 3). Therefore, the addition of sucrose at both 0.1 and 0.2 M to the freezing media containing Me_2SO at 5% significantly increased the GAG content in thawed cells.

DISCUSSION

Cryopreservation of somatic cells acquired much more relevance since the induction of pluripotency from such a cell type (Takahashi and Yamanaka, 2006). Once

somatic cells are transformed into pluripotent stem cells, they could be later differentiated onto the required cell type. The possibility of obtaining gametes from somatic cells is a huge challenge of enormous relevance for threatened and endangered species. However, such advanced biotechnology techniques are not widely developed. In this case, cryopreservation permits conserving the required cell type over the time until such techniques are more optimized. Therefore, in order to assure correct physiological responses from thawed cells, researchers have to develop appropriate and specific cryopreservation protocols.

The present study aimed at evaluating for the first time the ability of skin cells from the critically endangered European mink to be cryopreserved. To that end, researchers analyzed the impact of six cryopreservation media on different physiological properties of thawed cells such as the cell viability (by analyzing their membrane integrity) and the cell functionality (by measuring their metabolic activity, glycosaminoglycan synthesis and proliferative activity). Responses from thawed cells were compared to the control values obtained in fresh unfrozen ones.

The findings clearly showed several points concerning the cryopreservation of somatic cells from such a carnivore: Me_2SO at 10%, either alone or in combination with sucrose offers to the cells a suitable protection at both levels, viability as functionality after thawing, Me_2SO at 5% alone is not able to maintain high levels in the cellular physiological parameters analysed, the addition of sucrose at 0.1 or 0.2 M to the cryoprotectant media containing Me_2SO at 5%, significantly improved both viability as functionality of thawed cells, reaching values similar to those from cells cryopreserved in 10% Me_2SO and the favorable effect of adding sucrose to the freezing medium was only specific for the concentration of 5% Me_2SO.

The cryoprotective effect of Me_2SO usually increases with concentration but it can also cause toxic effects on the cells (Fahy, 1986). Me_2SO used at 10% v/v has provided protective effects on a wide variety of animal species (Wang *et al*., 2007; Sui *et al*., 2007; Cetinkaya and Arat, 2011) including human cells. The addition of a disaccharide to Me_2SO-based cryomedia is an approach used in several species (Erdag *et al*., 2002; Moritz and Labbe, 2008) to reduce the concentration of Me_2SO and thus prevent its potential toxic effects.

As for a wide variety of animal species, the findings showed that Me_2SO at 10% resulted to be suitable for preserving European mink somatic cells. Values from thawed cells for both viability and functionality were close to those from fresh unfrozen cells. Indeed, thawed cells exhibited viabilities around 80%, metabolic activities of 90% on average, GAG contents of about 85% and proliferative capabilities similar to those from fresh unfrozen cells. Likewise, the addition of sucrose (at both used concentrations) to the cryopreservant media containing 10% Me_2SO did not improve any results. Similar findings were previously observed in cells from the Iberian lynx (Leon-Quinto *et al*., 2011). Indeed when such cells were cryopreserved in Me_2SO at 10% either alone or along with sucrose (both at 0.1 or 0.2M) the three studied groups of thawed cells exhibited similar survival rates and metabolic activities were similar among the three studied groups of thawed cells (around 90 and 85%, respectively) not having the sucrose any effect. Regarding such an absence of effect from the sucrose, it should be noted that Me_2SO at 10% resulted in really high values for the analyzed physiological parameters. Therefore, it would be difficult to observe possible additional effects. In the present study, taking into account that functionality was evaluated 10 days after thawing, through three different physiological parameters as well as the absence of positive effect from the addition of sucrose, researchers can conclude that Me_2SO at 10% is a relevant cryoprotectant for skin cells from the European mink.

Considering a potential toxic effect from the Me_2SO, researchers also analyzed the cell protection when using half the concentration of this cryoprotectant (5%) either alone or along with sucrose. When the only cryoprotectant was Me_2SO at 5%, the survival rate of thawed cells was significantly reduced until about 50%. Therefore, Me_2SO at a low concentration of 5% did not provide adequate protection concerning cell viability after thawing. However, when we added sucrose both at 0.1 as 0.2 M to the freezing medium, the viability of the frozen-thawed cells significantly improved, reaching values similar to those from cells previously preserved in Me_2SO at 10%.

Such a partial protection from 5% Me_2SO observed immediately after thawing was ratified after a recovery period of 10 days. In fact, all the analyzed physiological parameters concerning functionality were significantly decreased 10 days after thawing when compared to the values obtained from fresh unfrozen cells and from thawed ones preserved in the rest of cryoprotectant media. As observed for viability when sucrose (either at 0.1 as 0.2 M) was added to the freezing media containing 5% Me_2SO, the three parameters used to estimate functionality increased up to levels similar to those obtained for Me_2SO at 10%. Thus, metabolic activity increased from 61±5 to near 90% on average, extracellular matrix content rose from 59±5 to about 80% and the proliferative activity of thawed cells was similar to that of fresh ones.

Such a similar cryoprotection provided by 5% Me_2SO along with a disaccharide when compared to that from Me_2SO at 10% has been already reported in stem cells (Seo *et al*., 2011). Indeed, thawed amniotic fluid-derived stem cells cryopreserved in solutions containing 5% Me_2SO and trehalose, exhibited similar results to those from the control (10% Me_2SO) in terms of culture growth, expression of cell surface antigens and mRNA expression of stem cell markers.

The mechanism by which the addition of sucrose causes such an enhanced protection is not clear. Intracellular ice formation is considered to be the primary mechanism by which cell damage occurs and cryoprotectants reduce the physical injury by preventing the formation of ice crystals. Permeating agents such as Me_2SO are thought to protect cells against freezing injury by reducing ice formation inside and outside the cells (Mazur, 1970). Sucrose is a non-permeating cryoprotectant and may provide protection in multiple ways (Crowe *et al*., 1988). It dehydrates cells and thus reduces the amount of cellular water prior to freezing. By dehydrating the cells, sucrose also helps to avoid excessive swelling and osmotic shock during removal of permeating cryoprotectants. In any case, it would be interesting to study in the future the underlying molecular mechanisms that could explain the interactions between low concentration of Me_2SO and sucrose.

CONCLUSION

This research represents the first study to perform a proper cryopreservation of somatic cells from the critically endangered European mink. The findings show procedures to maintain high success rates on both the viability and functionality of thawed skin cells. Such results are of relevance in order to provide properly cryobanked cells for the required application and when needed. At the same time, since skin biopsies can be easily and harmlessly collected, the results permit preserving a maximum of the population genetic diversity which could represent a complementary and preventive strategy within a wider conservation program for the endangered European mink. In addition, the findings could be also useful for other threatened species and mammals in general.

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