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Cryobanking the genetic diversity in the critically endangered Iberian lynx (*Lynx pardinus*) from skin biopsies. Investigating the cryopreservation and culture ability of highly valuable explants and cells [☆]

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ABSTRACT

Cryobanking skin samples permit preserving a maximum of genetic representation from the population biodiversity. This is a relevant aspect for threatened species, potentially menaced by an epizooty and from which it is difficult to obtain gametes. As a first step for properly cryobanking skin samples of a given species, the optimal conditions of culture and freezing have to be studied by covering a broad range of possibilities. This paper presents, for the first time, a systematic study of such conditions for the Iberian lynx (*Lynx pardinus*). To that end, we have analyzed twenty different culture conditions and fifteen different freezing solutions for skin explants, as well as three freezing solutions for isolated cells derived from them. The culture conditions included both two different culture strategies and several combinations of nutritional supplements and mitotic agents. For the freezing solutions, we have considered different concentrations of the permeating cryoprotectant dimethyl sulfoxide (Me₂SO) either alone (5%, 7.5%, 10%, 12.5% and 15% v/v for explants, 10% for isolated cells) or along with the non-permeating cryoprotectant sucrose (0.1 or 0.2 M). Our results have been analyzed through several quantitative parameters and show that only thawed explants cryopreserved in Me₂SO (10%) either alone or with sucrose (0.2 M) presented similar properties to those in optimal fresh cultures. In addition, for these freezing conditions, isolated thawed cells also presented high survival rates (90%) and percentages of cellular functionality (85%). These results, focussed on the most endangered felid in the world, could be also useful for other threatened/endangered species.

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Introduction

The Iberian lynx (*Lynx pardinus*) is the most endangered felid in the world [21] with about 200 individuals located in only three metapopulations, each isolated from each other. Since critically endangered species are vulnerable to catastrophic events such as epizooties [30], it is essential to preserve the current genetic diversity of the population. However, the banking of a genetic pool representative of the population biodiversity is very difficult in endangered species when only gametes are considered [25]. Through collection and processing of somatic tissues, we can in-

stead preserve the largest genetic biodiversity possible for both the male and female populations, as well as to avoid the irreversible biodiversity loss from individuals that die before reproducing. Cryobanking somatic tissues and cells could be therefore considered in threatened species as a preventive conservation strategy, based on the possible use of somatic cell nuclear transfer (SCNT) in case of a dramatic epizooty, as well as a supporting tool to develop studies addressed to favor the animal conservation [25,35,17,32,31,24]. Even if fundamental research in the field of SCNT is still needed [4,6] since the efficiency rate is presently very low, the previous step of processing and cryopreservation of somatic cells should be strongly taken into account in threatened species [24,25]. In this sense, skin fibroblasts are considered one of the most suitable cell types as nuclear donors [5]. In such a context of conservation biology, somatic tissues from endangered species are being preserved for Spain's endangered wildlife [24], Southern Africa's wildlife [1], the Frozen Zoo of San Diego [31,32] or the Frozen Ark Consortium (<http://www.frozenark.org>), among others.

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Skin biopsies can be harmlessly collected as a source of somatic tissues and cells but they have to be as small as possible in endangered species, in order to avoid any disturbance of the animals. Cryobanking and culture of highly valuable, scarce and small skin biopsies become therefore an important issue in order to recover them after thawing to be used when necessary.

In skin banking, one of the most widely used cryoprotectants either for tissues or cells is dimethyl sulfoxide (Me₂SO) [9,27,33,34,28], usually utilized alone or along with a non-permeating cryoprotectant. Me₂SO has been extensively used as a cryoprotectant because of its high membrane permeability, being even used as a control cryoprotectant when investigating new ones [33]. 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 [10]. Previous studies [33] in different cell types and tissues have demonstrated beneficial cryoprotective effects of sugars as trehalose, sucrose or D-allose. 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 [7]. Me₂SO and sucrose have been used in several species, like Goldfish [27,7] for cells and tissues, human dermal fibroblasts and human engineered-dermal tissues [8,34], human skin allografts [3] or lamb cartilage [29], among others. However, the results obtained are different depending on the species, experimental design and culture protocol.

Cryobanking tissues and cells are closely linked to cellular culture conditions. Nevertheless, little attention has been paid upon the ability of few and small explants from a given precious species to try to unfailingly outgrow cells. Two of the major factors influencing the success in cellular culture, as the culture strategy and composition of the medium, are usually studied separately. Explants were thus seeded either directly [14,15] or after a gentle collagenase pre-treatment [27,28]. Concerning the culture medium, it has been verified that the serum concentration has a significant influence on the cell growth [18,16,13], as well as mitotic agents like epidermal growth factor or fibroblast growth factor [2,12,20,26] which have been successfully used, especially in human skin explants.

At the present, neither the specific effect of any cryoprotectant on the viability of thawed Iberian lynx somatic tissues and cells nor the optimal specific cell culture protocol has ever been evaluated. In order to preserve a maximum of a population's genetic biodiversity and to dispose of somatic cells for developing any study addressed to favor the animal conservation, our global goal was to establish optimal culture and freezing procedures related to skin explants and cells from the Iberian lynx. For that, we firstly determined the optimal cellular culture conditions by considering simultaneously two culture strategies (non-treated and gentle collagenase pre-treated explants) and different concentrations of nutritional supplements as fibroblast growth factor, epidermal growth factor, glucose and foetal bovine serum, testing twelve culture conditions. Next, we assessed the ability of Iberian lynx skin explants and cells to be cryopreserved in different freezing solutions, by analyzing the effect of several concentrations of the permeating cryoprotectant Me₂SO used either alone or in combination with the non-permeating sucrose, studying fifteen freezing solutions for explants and three for isolated cells derived from them.

To our knowledge, no similar studies have been carried out neither in the Iberian lynx nor in any other felid. This work could therefore be useful for the rest of felids as well, at least as a starting point to initiate the implementation of Genetic Resource Banks, as the majority of the species of this family are threatened.

Materials and methods

Reagents

Culture media and supplements were purchased from Gibco/BRL (Grand Island, NY, USA), with the exception of collagenase, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and MTT assay which were from Sigma Chemical Co. (Madrid, Spain) and foetal bovine serum (FBS) from Biochrom (AG).

Skin sampling and preliminary treatment

The collection of skin biopsies was performed under agreement with the Environmental Council of the Regional Government of Andalusia. We implemented protocols to collect and send samples from living animals and provided sampling kits with the appropriate media [25]. 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. The samples consisted of millimetre-sized skin biopsies, which were isolated always profiting from animal manipulation for other reasons (radio tracking, sanitary check-ups, etc.).

Once in the laboratory the skin biopsies were washed several times in a washing medium, which consisted of D-MEM (Dulbecco's Modified Eagle's medium) supplemented with 25 mM HEPES, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 1% fungizone.

All the biopsies used for the present work were obtained from adult animals and taken outside the reproductive season.

Freezing and thawing procedures

In order to assess the cryopreservation ability of the Iberian lynx skin explants, we investigated the effect of several Me₂SO concentrations used either alone or in combination with sucrose. Based on the results obtained by Wang et al. [34], we selected five different Me₂SO concentrations (5%, 7.5%, 10%, 12.5% and 15% v/v). Each Me₂SO concentration was investigated alone and along with sucrose at 0.1 or 0.2 M, testing thus fifteen different freezing solutions for skin explants.

The cryoprotectant solutions were composed of D-MEM supplemented with 25 mM HEPES, 1% non-essential amino acids, 0.1 mM 2-mercaptoethanol, FBS at 35% (v/v) and the different cryoprotectant agents commented. According also to the results obtained by Wang et al. [34], the cryoprotectant solutions were added at 4 °C, at a cooling rate of 1 °C min⁻¹ until the cryovials reached -70 °C and subsequently plunged into liquid nitrogen. Based on the results obtained in this work for explants, cells derived from them were cryopreserved in freezing solutions containing as cryoprotectant agents Me₂SO at 10% v/v alone and together with sucrose at either 0.1 or 0.2 M, checking three different freezing solutions for isolated cells.

After at least two weeks of cryopreservation, tissues and cells were removed from liquid nitrogen. Briefly, cryovials were quickly thawed, maintained at room temperature in air for 0.5–1 min in order to avoid fracture in tissues, before plunging in a water bath at 37 °C. Thawed explants and cells were plated into appropriate media after washing of the cryoprotectant solution, which was diluted 10 times in basal culture medium (see the composition below).

Culture conditions

The basal culture medium was composed of D-MEM supplemented with 25 mM HEPES, 1% non-essential amino acids,

0.1 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/mL penicillin and 0.1 mg/mL streptomycin. In order to determine the optimal culture conditions we tested in fresh explants different nutritional supplements and culture strategies: (1) Nutritional supplements. As basic supplements, we analyzed the effects on the cellular culture of two glucose concentrations, both a low 1 g/L and a high 4.5 g/L value, each one used together with one of the three studied percentages of FBS (10%, 15% or 20%). Such supplements were added to the basal medium in all possible combinations. For the combination giving the best results, we also analyzed the possibility of obtaining a further amelioration by adding the mitotic agents fibroblast growth factor (FGF) or EGF, at 5 and 10 ng/ml each one; (2) Culture strategies. After proper washing, each fresh biopsy was cut into two pieces of similar size by using a scalpel. The two resulting parts were then submitted to one of the two following culture strategies: (a) Non-treated explants: half of each biopsy was directly seeded without suffering any treatment; (b) Gentle collagenase pre-treated explants: the other half from the same biopsy underwent a gentle collagenase treatment, which was focused on studying a possible amelioration on explants culture, as previously observed in other animal species [27,28]. Explants were thus incubated at 37 °C for 30 min in 0.2 mg/mL of collagenase diluted in the washing medium, rinsed several times afterwards and immediately plated.

In all cases, the explants were transferred onto gelatine-coated 35 mm Petri dishes (Corning, NY) in basal culture medium supplemented with the mentioned nutritional supplements and incubated in 5% CO₂ in air and 95% relative humidity. Searching for a maximum of adhesion, explants were cultured for 2 h at 37 °C in 5% CO₂ in air before adding the culture medium [25,24,23], which was changed every 2–3 days.

Assessment of explants culture quality

Culture quality was assessed and quantified in fresh and thawed explants according to different parameters: (1) plating efficiency (PE), measured as the percentage of cultures with tissue attached at day 1 after seeding; (2) cell-donor (CD) explants, calculated as the percentage of the cultures with outgrowing cells around explants at day 3 after seeding; (3) population doubling (PD) rate, by the population doubling assay, which was calculated as follows: skin cells around explants were allowed to proliferate for 7 days, trypsinized at such a day, counted and seeded on new gelatine-coated dishes (day 0) until seven days later (day 7), when the cells were trypsinized again and recounted. The PD rate was calculated by using the following equation [18,22]: $PD = \log_2 (\text{Cell number at Day 7}) / (\text{Cell number at Day 0})$. Viable cells were counted on a hemocytometer using the trypan blue exclusion assay.

In thawed tissues we introduced an additional parameter, the cellular rate (CR) around explants, calculated as the percentage of cells outgrowing around them at day 7 after thawing with respect to the cells obtained in the same fresh ones at the same time lapse (day 7 after plating).

Cell viability assessment

Cells obtained from fresh explants were trypsinized at day 7 after plating. Once isolated, living cells were counted, pooled and subdivided into four groups: one group was used as the fresh control and the other three were frozen in the three freezing media commented above.

The viability of thawed cells was analyzed by determining their membrane integrity and functionality, using the trypan blue staining and the MTT methods, respectively: (a) the survival rate (SR) was calculated by means of the trypan blue staining, as the ratio of the number of cells presenting intact membranes after thawing

to those cryopreserved x 100; (b) the cellular functionality (CF) of viable 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, isolated fresh and thawed living cells were seeded and cultured in a humidified atmosphere of 5% CO₂ at 37 °C. After 6 h, 20 µL MTT solution (5 mg/mL in PBS) were added and the plates were incubated for an additional 4 h at 37 °C. To achieve solubilization of formazan crystals formed in viable cells, MTT solubilization solution (200 µL) was added and the absorbance was then measured at a wavelength of 570 nm. For comparison, the fresh-control group was assayed for cell functionality and the mean value obtained was normalized to 100%, which was used as the reference for the cryopreserved cells.

Statistical analysis

To assess the cellular culture quality we analyzed the possible effect on three target variables (plating efficiency, cell-donor explants and population doubling rate) of three factors: culture strategy (two levels, non-treated and gentle collagenase pre-treated explants), glucose concentration (two levels, 1 and 4.5 g/L), and FBS percentage (three levels, 10%, 15% and 20%). A multi-factor ANOVA was applied with two factors: FBS (three levels) and glucose (two levels) and the corresponding interaction between them. An additional study of the influence of the FBS factor on the target variable PE was carried out with a single-factor ANOVA. The analysis of the mean behavior of the variable PE between the levels of glucose was made with a Student *t*-test. In the case of comparison between the two levels of glucose, tests for the equality of two proportions were applied. The cell-donor explants (CD) variable was studied by using contingency tables and chi-Square tests. The population doubling (PD) variables were analyzed 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. In order to detect statistical differences, a *P* < 0.05 level of significance was applied in all the considered analyses. Each experiment was repeated at least four times.

Results

Establishment of optimal culture conditions

Optimal cellular culture conditions were developed in fresh tissues by analyzing the effect of the previously mentioned two culture strategies and the addition of several concentrations of Epidermal Growth Factor, Fibroblast Growth Factor, glucose and foetal bovine serum, testing twelve different treatments.

Table 1 summarizes the results obtained for the PE, CD and PD parameters in all the combinations of basic supplements, foetal bovine serum and glucose, analyzed in both culture strategies. This Table shows that the PE parameter was 100% in all the cases. Such a complete success in PE was likely due to the culture of explants for 2 h at 37 °C in 5% CO₂ in air before adding the culture medium, a pre-treatment applied in all cellular cultures.

Concerning the CD parameter, at day 3 after seeding we found that outgrowing cells around explants had an overall trend of appearing before in collagenase pre-treated explants than in non-treated ones (see Table 1). In this way, a percentage of 100% was

Table 1
Assessment of the effect of basic supplements (glucose and foetal bovine serum) on fresh explants culture.

Culture strategy	FBS (%)	Glucose (g/L)	PE (%)	CD (%)	PD
(a) Non-treated explants	10	1	100	25 ^a	1.28 ± 0.12 ^a
		4.5	100	50 ^b	2.39 ± 0.48 ^b
	15	1	100	75 ^c	3.11 ± 0.62 ^b
		4.5	100	100 ^d	4.47 ± 0.25 ^c
	20	1	100	50 ^b	3.69 ± 0.27 ^b
		4.5	100	100 ^d	4.26 ± 0.31 ^c
(b) Gentle collagenase pre-treated explants	10	1	100	75 ^c	1.34 ± 0.17 ^a
		4.5	100	100 ^d	2.57 ± 0.47 ^b
	15	1	100	100 ^d	3.13 ± 0.36 ^b
		4.5	100	100 ^d	4.74 ± 0.55 ^c
	20	1	100	75 ^c	3.38 ± 0.71 ^b
		4.5	100	100 ^d	4.82 ± 0.48 ^c

The culture quality assessment was analyzed by the parameters: plating efficiency (PE), calculated as the percentage of the cultures with tissue attached at day 1 after seeding; cell-donor (CD) explants, calculated as the percentage of the explants with outgrowing cells around them at day 3; and population doubling (PD) rate, calculated by the population doubling assay in a time lapse of 7 days starting at day 7 after plating. Data are percentage or mean ± S.E.M. Each treatment was repeated four times. Within columns values with different superscripts (a,b,c) are significantly ($P < 0.05$) different.

observed in 4 out of the 6 combinations in collagenase pre-treated explants, while only in 2 out of 6 was this observed for non-treated explants. However, after 7 days all the explants presented cells around them in whichever strategy employed.

The most discriminating parameter was found to be the PD rate, computed as described in Materials and methods. We found a significant dependence of the PD rate on the glucose concentration at any fixed FBS concentration and in any culture strategy (see Table 1). Indeed, the use of a higher glucose concentration always resulted in a significant increase in the PD rate. The FBS percentage had also some significant effects on the PD rate. For both culture strategies, the minimum PD value was obtained for FBS at 10% along with a low glucose concentration. In the opposite side, the maximum PD rate was obtained with FBS at either 15% or 20% in combination with a high glucose concentration, without significant differences between both percentages of FBS (15% or 20%) in either culture strategies. However, the culture strategy had a non-significant impact on the PD rate for any combination of FBS and glucose.

Since FBS at 20% in the culture medium did not induce any significant increase as compared to FBS at 15%, we choose as the best result a culture medium supplemented with FBS at 15% and a high (4.5 g/L) glucose concentration as basic complements. Using such culture conditions, we then studied a possible potentiating effect from the use of the mitogenic agents FGF and EGF. When FGF and EGF were individually added to the culture media the percentages of PE and CD were maintained as in the control, being thus 100% for both parameters in all the combinations studied. Once again, the PD rate resulted in being the most discriminating parameter; although significant differences were found solely for non-treated explants (see Table 2). Indeed, the averaged PD value obtained for non-treated explants with EGF at 10 ng/mL was significantly higher (5.21 ± 0.28) than those obtained in all other cases including the control culture (4.47 ± 0.25, $P = 0.035$), FGF at either 10 ng/mL (4.61 ± 0.35, $P = 0.046$) or 5 ng/mL (4.40 ± 0.31, $P = 0.049$) and EGF at 5 ng/mL (4.54 ± 0.31, $P = 0.048$). Instead, no significant differences were observed in pre-treated explants at any concentration of added FGF or EGF with respect to its specific control value without growth factor.

Summarizing, our results favor the culture of tissues without any collagenase pre-treatment, and using a culture medium composed by the basal medium supplemented along with FBS at 15%, glucose at 4.5 g/L and EGF at 10 ng/mL. The fact of using non-treated

Table 2
Assessment of the effect of the mitotic agents fibroblast growth factor (FGF) and epidermal growth factor (EGF) on fresh explants culture.

Culture strategy	Growth factor	Concentration (ng/mL)	PD
(a) Non-treated explants	GF(control)	–	4.47 ± 0.25 ^a
		5	4.40 ± 0.31 ^a
	EGF	10	4.61 ± 0.35 ^a
		5	4.54 ± 0.31 ^a
		10	5.21 ± 0.28 ^b
		10	5.21 ± 0.28 ^b
(b) Gentle collagenase pre-treated explants	GF(control)	–	4.74 ± 0.55 ^a
		5	4.72 ± 0.64 ^a
	EGF	10	4.95 ± 0.46 ^a
		5	4.63 ± 0.51 ^a
		10	5.12 ± 0.77 ^a
		10	5.12 ± 0.77 ^a

FGF and EGF were added to the basal medium supplemented with FBS 15% and glucose 4.5 g/L. Since the plating efficiency (PE) and the cell-donor (CD) explants were 100% in all cases, the only parameter shown in the table is the population doubling (PD) rate. Data are percentage or mean ± S.E.M. Each treatment was repeated four times. Within column and considering separately each culture strategy, values with different superscripts (a,b) were considered significantly different ($P < 0.05$) when compared to its respective control value.

explants has the additional advantage of avoiding unnecessary enzymatic damage upon the tissues and, therefore, they could be available for additional freezing and thawing processes. 359 360 361

Ability of Iberian lynx skin explants to be cryopreserved: culture quality in thawed explants 362 363

We evaluated the ability of thawed explants to be cultured after cryopreservation in fifteen different freezing solutions, by comparing the values obtained in the parameters quantifying the culture quality with those obtained in fresh ones. In order to avoid an excessive number of variables, the culture conditions were always those favored as the optimal ones in the previous subsection: non-treated explants cultured in a basal medium supplemented along with FBS at 15%, glucose at 4.5 g/L and EGF at 10 ng/mL. In this way, our analysis will be now focused on the possible effects of the different freezing solutions. 364 365 366 367 368 369 370 371 372 373

When considering the PE parameter, the complete attachment observed in fresh controls was observed in frozen-thawed explants as well (see Table 3). The parameter CD, indicating the percentage 374 375 376

Table 3
Assessment of Iberian lynx skin explants' ability to be cryopreserved.

Me ₂ SO (%)	Sucrose (M)	PE (%)	CD (%)	CR (%)	PD
5	0	100	25 ^a	58 ± 11 ^a	2.11 ± 0.37 ^b
		100	25 ^a	59 ± 19 ^a	2.21 ± 0.42 ^b
	0.1	100	50 ^b	63 ± 21 ^a	2.96 ± 0.31 ^b
		100	75 ^c	64 ± 14 ^a	2.76 ± 0.41 ^b
	0.2	100	50 ^b	65 ± 16 ^a	2.81 ± 0.34 ^b
		100	75 ^c	65 ± 15 ^a	3.74 ± 0.36 ^b
7.5	0	100	75 ^c	76 ± 13 ^a	5.01 ± 0.39 ^a
		100	75 ^c	75 ± 19 ^a	4.49 ± 0.26 ^b
	0.1	100	75 ^c	79 ± 16 ^a	5.08 ± 0.32 ^a
		100	75 ^c	71 ± 9 ^a	3.54 ± 0.56 ^b
	0.2	100	75 ^c	75 ± 15 ^a	3.41 ± 0.48 ^b
		100	75 ^c	77 ± 12 ^a	4.23 ± 0.37 ^b
15	0	100	75 ^c	73 ± 16 ^a	3.62 ± 0.41 ^b
		100	50 ^b	69 ± 11 ^a	3.54 ± 0.38 ^b
	0.1	100	50 ^b	68 ± 14 ^a	3.91 ± 0.52 ^b
		100	50 ^b	68 ± 14 ^a	3.91 ± 0.52 ^b
	0.2	100	100	100	5.21 ± 0.28 ^a
		100	100	100	5.21 ± 0.28 ^a

The culture success was analyzed in thawed explants by: plating efficiency (PE), cell-donor (CD) explants, cellular rate (CR) around explants, and population doubling (PD) rate. Data are percentage or mean ± S.E.M. Each treatment was repeated four times. The control values refer to those obtained in fresh non-treated explants cultured in FBS at 15%, glucose at 4.5 g/L and EGF at 10 ng/mL. Within columns values with different superscripts (a,b) are considered significantly ($P < 0.05$) different.

of explants presenting outgrowing cells at day 3, was lower in frozen-thawed explants than in fresh ones, not reaching 100% at any case. However, in thawed explants the values of such a parameter still achieved a high percentage of 75% in 9 out of the 15 freezing media (Table 3), particularly in all the explants cryopreserved in Me₂SO at 10% and 12.5% v/v, either with or without added sucrose. After 7 days, all the explants presented cells around them, however. By means of the CR parameter, we calculated the percentage of cells outgrowing around thawed explants at day 7 respect to those obtained in the same fresh ones at the same time lapse. Such a CR parameter was clearly decreased in all thawed explants from whatever the freezing solution used (Table 3), with no statistically significant differences among them. Noteworthy however, as observed for the CD parameter, that all the explants cryopreserved in Me₂SO at 10% and 12.5% v/v, either with or without added sucrose, achieved in absolute values the highest cellular percentage, being around 65–75% respect to the fresh controls. The proliferation ability of cells outgrowing around the thawed tissues was estimated by the PD rate once isolated. As Table 3 shows, we found a significant decrease of this parameter for the majority of the thawed explants, with the exception of those previously cryopreserved in Me₂SO at 10% v/v either alone or along with sucrose at

0.2 M. In these two latter cases, the achieved PD rates were similar to those observed in the fresh controls.

Viability of thawed cells derived from skin biopsies of the Iberian lynx

The viability of isolated thawed cells previously obtained from skin explants cultures was evaluated by means of the survival rate and the percentage of cellular functionality. As shows Fig. 1, the survival rates after thawing were very high and around 90% for cells from the three freezing solutions used, with no statistically significant difference among them.

Concerning the percentage of cellular functionality, living cells previously cryopreserved in the three freezing media presented after thawing mean values around 85%, without any statistically significant difference among the three groups (see Fig. 2).

Discussion

Cryobanking skin tissues and cells permit maintaining a maximum representation from the population biodiversity. This is a relevant aspect to take into account in endangered and threatened species, populations potentially menaced by an eventual epizooty and from which it is difficult to obtain gametes. However, properly cryobanking highly valuable, scarce and small tissues and derived cells require developing appropriate protocols. In order to establish optimal culture and freezing procedures related to skin tissues and cells from the Iberian lynx, the most endangered felid in the world, we analyzed twelve different culture conditions and fifteen different freezing solutions for skin explants, as well as three different freezing solutions for isolated cells derived from them. We selected appropriate quantitative parameters, which conduced to discern among all the possibilities studied, those presenting the best conditions of culture and freezing. Therefore, by covering a broad spectrum of possibilities we established in this work the optimal conditions to implement a genetic resource bank (GRB) by means of skin tissues and cells from an endangered species when gametes to preserve are very scarce, a common problem when dealing with endangered species.

Skin samples in endangered and threatened species have to be as small as possible, in order to avoid any disturbance of the animals, what constitutes a problem to overcome when trying to assure the success in the cellular culture. In highly valuable and small explants, the establishment of the optimal culture conditions becomes therefore an important issue. With such a goal, we considered analyzing the effect of several nutritional supplements and culture strategies. Among the nutritional supplements, we chose those susceptible of having a relevant effect upon the somatic cell cultures, differentiating between basic (glucose and serum) and additional (growth factors) supplements. Concerning the basic supplements, the serum concentration used in the culture medium has a significant influence on the cell growth [18]. A serum percentage of 10% has probably been the most widely used in previous studies [27,33,28,18,13]. In our work, we were interested in analyzing a possible potentiating effect induced by higher serum concentrations. We hence compared the effect of several foetal bovine serum (FBS) percentages: 10%, 15% and 20%. On the other hand, cells require a constant energy source to drive metabolic processes, being carbohydrates, especially D-glucose, the key energy additives in culture media. We also therefore considered interesting to compare the possible effects on the cellular growth of the glucose concentration. To that end, each FBS percentage studied was combined with either a low (1 g/L) or high (4.5 g/L) glucose concentration by using two culture strategies, non-treated and gentle collagenase pre-treated explants, testing thus twelve different culture conditions.

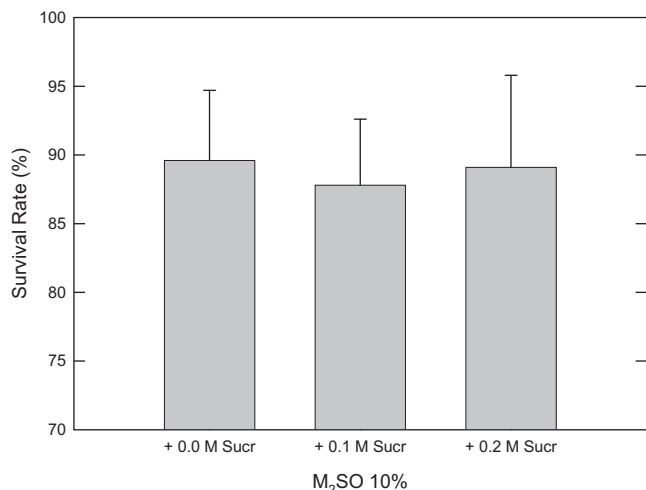


Fig. 1. Effects of different cryoprotectants' concentrations on the cellular survival rate after thawing.

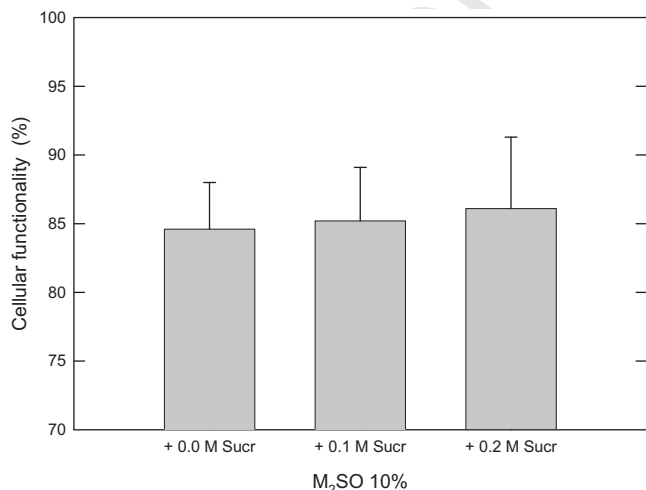


Fig. 2. Effects of different cryoprotectants' concentrations on the cellular functionality after thawing.

In order to establish the optimal culture conditions for skin explants we quantified the culture quality by means of the parameters plating efficiency (PE), cell-donor (CD) explants and population doubling (PD) rate. The former PE parameter has been widely used in the literature in assessing culture quality, mainly because it is the first requirement to permit the cell outgrowth around explants. We achieved complete success in such a parameter both for fresh and thawed explants, probably due to their pre-treatment that consisted of culturing for 2 h at 37 °C in 5% CO₂ in air before adding the culture medium, conditions previously established in our laboratory [25,24,23]. Concerning the CD parameter, outgrowing cells around explants had an overall trend of appearing in collagenase pre-treated explants before than in non-treated ones. However, after 7 days all the explants presented cells around them in whichever the strategy employed. Proliferation ability of the cells growing around explants in the different culture conditions was quantitatively assessed by the population doubling (PD) assay. Since we achieved 100% in PE and all the explants presented cells around them after 7 days of culture, the PD rate acquired more relevance than the other two parameters, in order to be able to discern the optimal culture conditions among the twelve tested. The relevance of using a high glucose concentration was objectivized when comparing the PD rate values. Indeed, in both culture strategies a significant improvement in the PD rate was found at any FBS percentage when the glucose concentration was changed from a low to a high value. A significant amelioration in the proliferation ability of the cells was also obtained by increasing the FBS percentage up to 15% or 20%, as compared to the most widely used percentage of 10%. Therefore, our results showed that the use of high glucose (4.5 g/L) concentration and high FBS percentage, either 15% or 20%, led to achieve the best results in the cellular cultures, in whichever of the two strategies employed. Likewise, FBS at 20% in the culture medium did not induce any significant increase as compared to FBS at 15%, being therefore unnecessary to use the former percentage. With the finality of trying an additional positive effect on the already high performance obtained for the cell cultures in presence of glucose at 4.5 g/L and FBS at 15%, we added the mitogenic agents EGF or FGF at 5 and 10 ng/mL each one. As a result, the high PD rate obtained by means of modulating the basic supplements studied was significantly increased but only in non-treated explants with EGF added at 10 ng/mL.

Summarizing, our results showed that no single strategy in the culture of explants stood out against any other. We chose therefore the “non-treated explants” strategy since the PD rate significantly increased when EGF was added at 10 ng/mL and so that explants can be utilized several times, avoiding possible enzymatic damage to the tissue. We hence delimited the optimal culture conditions to apply in skin explants culture, which include as supplements glucose at 4.5 g/L, FBS at 15% and EGF at 10 ng/mL by using non-treated explants as culture strategy.

Once the optimal culture conditions for skin samples from the Iberian lynx were established, we next analyzed the effects of different freezing solutions on the explants and cell culture quality after thawing. Because of its high membrane permeability, Me₂SO has been extensively used as a protectant for cryopreservation of different types of cells and tissues, i.e., human platelets [11], umbilical cord blood cells [19] or human skin allografts [3], among others. However, despite the protection that the cryoprotectant offered to the cells during freezing and thawing, cryoprotectants can be damaging to cells, especially when they are used in high concentrations [10]. In this way, Wang et al. [34] showed that exposure of dermal fibroblast to Me₂SO at concentrations lower than 15% (v/v) leads to better cell survival rates, being >90% when Me₂SO was used at 5 and 10%, regardless of the duration and tem-

perature at which exposure occurred. The same authors also showed that when using Me₂SO at 10% (v/v) and a cooling rate of 1 °C min⁻¹ (from 4 to -60 °C), the frozen-thawed engineered-dermal tissues could retain the maximal cell viability, which was 75% of the fresh control viability. Considering these previous works we chose a cooling rate of 1 °C min⁻¹ and Me₂SO concentrations around 10 but lower than 15% (5%, 7.5%, 10%, 12.5% and 15% v/v) for explant cryopreservation. On the other hand, the combination of dimethyl sulfoxide and non-permeating cryoprotectant sucrose has been previously studied. When Goldfish fin cells were cryopreserved by using dimethyl sulfoxide at 13% v/v together with sucrose at 166 mM, half of them survived after thawing [27]. In tissues, when Me₂SO was used to preserve Goldfish fins at 10% v/v in combination with sucrose at 125 mM, the frozen-thawed explants showed after 14 days of culture the same cell growth rate and somatic cell number as the fresh ones [28].

Considering the previous related works, we investigated the ability of Iberian lynx skin explants and cells to be cryopreserved by means of Me₂SO either with or without added sucrose. Fifteen different freezing solutions were studied for explants and three for isolated cells. The assessment of the cryopreservation effects was carried out by comparing the development of the cellular culture in thawed versus fresh explants through the previously commented quantitative parameters PE, CD and PD. One additional parameter was introduced in this part of the work, the cellular rate (CR), which informed us about the relative percentage of cells outgrowing around thawed explants at day 7 after thawing, with respect to the cells obtained in fresh ones at the same time lapse.

Our results showed that the plating efficiency (PE) of thawed explants was not altered by any of the freezing solutions. The percentage of explants presenting outgrowing cells at day 3 (CD parameter) in frozen-thawed explants was instead moderately lower than in fresh ones, not reaching 100% in any case. In the same way, the CR parameter at day 7 was decreased in all thawed explants. Considering both parameters, CD and CR, all explants cryopreserved in Me₂SO at 10% and 12.5% v/v, either with or without added sucrose, achieved the highest percentages being them similar for the six freezing conditions. Once again, as occurred in fresh explants when comparing the culture conditions, the population doubling rate was the quantitative parameter conducting to clearly differentiate among the different freezing solutions. The ability to proliferate of the cells outgrowing around explants was, once isolated, significantly decreased in the majority of the thawed explants. However, in spite of the delay observed in thawed explants in appearing cell outgrowing around explants and even a decrease in the cell number at day 7 after thawing, cells coming from tissues cryopreserved in Me₂SO either alone or along with sucrose 0.2 M achieved population doubling rates similar to those found for fresh ones.

In order to freeze isolated cells obtained from explants cultures we were interested in assessing not only the number of living cells remaining after thawing but also their functionality. Survival rates obtained after thawing were very high for cells from the three freezing solutions used, the mean value being around 90% and thus similar to those obtained in other species when the cryoprotectant used was Me₂SO at 10% alone [33,34]. Moreover, the three freezing solutions used, Me₂SO alone as well as in combination with sucrose at either 0.1 or 0.2 M, resulted in retaining about 85% of the cellular activity in thawed living cells.

Taken together the results obtained from thawed tissues and cells, the cryoprotectant Me₂SO at 10% v/v, either alone or along with the non-permeating cryoprotectant sucrose at 0.2 M, properly preserves the culture properties of skin explants. Likewise, Me₂SO at 10% v/v alone and together with sucrose at 0.1 or 0.2 M seem to be very appropriate to cryopreserve isolated skin cells.

Conclusions

This study represents the first attempt for the felid family in general and the Iberian lynx in particular, to delimit the requirements and conditions for properly cryobanking skin explants and cells. Our results are relevant to establish optimal culture and freezing procedures related to somatic tissues and cells, as a first step to initiate a genetic resource bank from skin biopsies when gametes are difficult to obtain. Considering that the majority of the species of the felid family are threatened and that no similar studies have been carried out in any other species of this family, our results could provide a useful starting point to develop genetic resource banks for other threatened/endangered felids, in order to cryopreserve their genetic patrimony.

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References

- [1] P. Bartels, A. Kotze, Wildlife biomaterials banking in Africa for now and the future, *J. Environ. Monitor.* 8 (2006) 779–781.
- [2] F.Y. Bhora, B.J. Dunkin, S. Batzri, H.M. Aly, B.L. Bass, Y.A.N. Sidaw, J.W. Harmon, Effect of growth factors on cell proliferation and epithelialization in human skin, *J. Surg. Res.* 59 (1995) 236–244.
- [3] D. Bravo, T.H. Ringley, N. Gibran, D.M. Strong, H. Newman-Gage, Effect of storage and preservation methods on viability in transplantable human skin allografts, *Burns* 26 (2000) 367–378.
- [4] K.H.S. Campbell, R. Alberio, I. Choi, P. Fisher, R.D.W. Kelly, J.-H. Lee, W. Maalouf, Cloning: eight years after Dolly, *Reprod. Domest. Anim.* 40 (2005) 256–268.
- [5] K.H.S. Campbell, P. Fisher, W.C. Chen, I. Choi, R.D.W. Kelly, J.-H. Lee, J. Xhu, Somatic cell nuclear transfer: past, present and future perspectives, *Theriogenology Suppl.* 68 (2007) S214–31.
- [6] J. Cibelli, A decade of cloning mystique, *Science* 316 (2007) 990–992.
- [7] J.H. Crowe, L.M. Crowe, J.F. Carpenter, A.S. Rudolph, C.A. Wistrom, B.J. Spargo, T.J. Anchordoguy, Interactions of sugars with membranes, *Biochim. Biophys. Acta* 947 (1988) 367–384.
- [8] R.A. Dawn, L. Kang, J. Mansbridge, Practical consideration for large-scale cryopreservation of a tissue engineered human dermal replacement, *Adv. Heat Mass Transfer Biotechnol.* 44 (1999) 77–91.
- [9] G. Erdag, A. Eroglu, J.R. Morgan, M. Toner, Cryopreservation of fetal skin is improved by extracellular trehalose, *Cryobiology* 44 (2002) 218–228.
- [10] G.M. Fahy, The relevant of cryoprotectant toxicity to cryobiology, *Cryobiology* 23 (1986) 1–13.
- [11] D.Y. Gao, K. Neff, H.Y. Xiao, H. Matsubayashi, X.D. Cui, P. Bonderman, K. Harvey, J.A. McIntyre, J. Critser, C.C. Miraglia, T. Reid, Development of optimal techniques for cryopreservation of human platelets. I. Platelets activation during cold storage (at 22 and 8 °C) and cryopreservation, *Cryobiology* 38 (1999) 225–235.

- [12] S. Gibbs, A.N. Silva-Pinto, S. Murli, M. Huber, D. Hohl, M. Ponec, Epidermal growth factor and keratinocyte growth factor differentially regulate epidermal migration, growth and differentiation, *Wound Repair Regen.* 8 (2000) 192–203.
- [13] M.D. Goissis, H.V.A. Caetano, M.G. Marques, F.R.O. de Barros, W.B. Feitosa, M.P. Milazzotto, M. Binelli, M.-E.O.A. Assumpção, J.A. Visintin, Effects of serum deprivation and cycloheximide on cell cycle of low and high passage porcine fetal fibroblasts, *Reprod. Domest. Anim.* 42 (2007) 660–663.
- [14] Z.M. Han, D.Y. Chen, J.S. Li, Q.Y. Sun, P.Y. Wang, J. Du, H.M. Zhang, Flow cytometric cell-cycle analysis of cultured fibroblasts from the giant panda, *Ailuropoda melanoleuca*, *Cell Biol. Int.* 27 (2003) 349–353.
- [15] M.A. Hashem, D.P. Bhandari, S.K. Kang, B.C. Lee, H.W. Suk, Cell cycle analysis of in vitro cultured goral (*Naemorhedus caudatus*) adult skin fibroblast, *Cell Biol. Int.* 30 (2006) 698–703.
- [16] H. Hashimoto, H. Toyohara, H. Yokoyama, M. Sakaguchi, K. Ozato, Y. Wakamatsu, Effects of carp serum on the growth of goldfish fin cells in early passage, *J. Fish Biol.* 50 (1997) 201–207.
- [17] W.V. Holt, P.M. Bennett, V. Volobouev, P.F. Watson, Genetic resource banks in wildlife conservation, *J. Zool.* 238 (1996) 531–544.
- [18] M. Hosokawa, H. Fijisawa, Z. Bing-Hua, H. Jujo, K. Higuchi, In vitro study of the mechanisms of senescence acceleration, *Exp. Gerontol.* 32 (1997) 197–203.
- [19] C.J. Hunt, S.E. Armitage, D.E. Pegg, Cryopreservation of umbilical cord blood: 1. Osmotically inactive volume, hydraulic conductivity and permeability of CD34(+) cells to dimethyl sulphoxide, *Cryobiology* 46 (2003) 61–75.
- [20] M. Huttunen, M. Hyttinen, G. Nilsson, J.H. Butterfield, M. Horsmanheim, I.T. Harvima, Inhibition of keratinocyte growth in cell culture and whole skin culture by mast cell mediators, *Exp. Dermatol.* 10 (2001) 184–192.
- [21] IUCN, IUCN Red List of threatened species, 2008, [<http://www.iucnredlist.org>].
- [22] K.R. Kranc, S.D. Bamforth, J. Bragança, C. Norbury, M.V. Lohuizen, S. Bhattacharya, Transcriptional coactivator cited2 induces Bmi1 and Me118 and controls fibroblast proliferation via Ink4a/ARF, *Mol. Cell Biol.* 23 (2003) 7658–7666.
- [23] T. León-Quinto, J. Jones, A. Skoudy, M. Burcin, B. Soria, In vitro directed differentiation of mouse embryonic stem cells into insulin-producing cells, *Diabetologia* 47 (2004) 1442–1451.
- [24] T. León-Quinto, J. Jones, B. Soria, B. Nuevas estrategias de biotecnología para la conservación de fauna amenazada: los bancos celulares somáticos y su aplicación al quebrantahuesos, in: A. Margalida, R. Heredia (Eds.), *Biología de la conservación del quebrantahuesos *Gypaetus barbatus* en España*, Parques Nacionales, Publicaciones del Ministerio de Medio Ambiente, Madrid, 2005, pp. 305–314.
- [25] T. León-Quinto, M.A. Simón, R. Cadenas, J. Jones, F. Martínez-Hernández, J.M. Moreno, A. Vargas, F. Martínez, B. Soria, Developing biological resource banks as a supporting tool for wildlife reproduction and conservation, *Anim. Reprod. Sci.* 112 (2009) 347–361.
- [26] T. Li, L. Lu, Epidermal growth factor-induced proliferation requires down-regulation of pax6 in corneal epithelial cells, *J. Biol. Chem.* 280 (2005) 12988–12995.
- [27] P.E. Mauger, P.Y. Le Bail, C. Labbé, Cryobanking of fish somatic cells: optimizations of fin explant culture and fin cell cryopreservation, *Comp. Biochem. Physiol.* 144 (2006) 29–37.
- [28] C. Moritz, C. Labbe, Cryopreservation of goldfish fins and optimization for fin scale cryobanking, *Cryobiology* 56 (2008) 181–188.
- [29] D.E. Pegg, L. Wang, D. Vaughan, C.J. Hunt, Cryopreservation of articular cartilage. Part 2: mechanisms of cryoinjury, *Cryobiology* 52 (2006) 347–359.
- [30] M.E. Roelke, W.E. Johnson, J. Millán, F. Palomares, E. Revilla, A. Rodríguez, J. Calzada, P. Ferreras, L. León-Vizcaíno, M. Delibes, S.J. O'Brien, Exposure to disease agents in the endangered Iberian lynx (*Lynx pardinus*), *Eur. J. Wildl. Res.* 54 (2008) 171–178.
- [31] O. Ryder, Conservation genomics: applying whole genome studies to species conservation efforts, *Cytogenet. Genome Res.* 108 (2005) 6–15.
- [32] O. Ryder, A. McLaren, S. Brenner, Y.P. Zhang, K. Benirschke, DNA banks for endangered animal species, *Science* 288 (2000) 275–277.
- [33] L. Sui, R. Nomura, Y. Dong, F. Yamaguchi, K. Izumori, M. Tokuda, Cryoprotective effects of D-allose on mammalian cells, *Cryobiology* 55 (2007) 87–92.
- [34] X. Wang, T.C. Hua, D.W. Sun, B. Liu, G. Yang, Y. Cao, Cryopreservation of tissue-engineered dermal replacement in Me₂SO: toxicity study and effect of concentration and cooling rates on cell viability, *Cryobiology* 55 (2007) 60–65.
- [35] D.E. Wildt, Genetic Resource Banks for conserving wildlife species. justification, examples and becoming organized on a global basis, *Anim. Reprod. Sci.* 28 (1992) 247–257.