Cryobiology 68 (2014) 227–233

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Different cryopreservation requirements in foetal versus adult skin cells from an endangered mammal, the Iberian lynx (*Lynx pardinus*) ☆

Trinidad León-Quinto [a,*], Miguel A. Simón [b], Rafael Cadenas [b], África Martínez [a], Arturo Serna [c]

[a] *Institute of Bioengineering, University Miguel Hernández, Av. Universidad s/n, 03202 Elche, Spain*
[b] *Environmental Council of the Regional Government of Andalusia, C/Av., Manuel Siurot 50, 41071 Sevilla, Spain*
[c] *Department of Physics and Computer Architecture, Area of Applied Physics, University Miguel Hernández, Av. Universidad s/n, 03202 Elche, Spain*

A R T I C L E I N F O

Article history:
Received 4 November 2013
Accepted 4 February 2014
Available online 12 February 2014

Keywords:
Felid
Mammal
Cryopreservation
Induced pluripotency
Somatic cell
Fibroblast
Genome resource bank
Conservation strategy
Population's genetic biodiversity
Threatened species

A B S T R A C T

Cryobanking somatic foetal cells acquire much relevance in endangered species for biodiversity conservation purposes. Such cells could be later used to reintroduce the lost genes into the breeding pool, by inducing pluripotency and/or nuclear transfer if necessary. Since requirements for preserving foetal cells are not always the same as for adult ones, we evaluated the cryosensitivity of foetal skin cells in comparison with adult ones from the critically endangered Iberian lynx. Responses to cryoinjury were analyzed in both thawed cell types by means of cell viability and functionality (by analyzing their membrane integrity, metabolic activity, glycosaminoglycan content and proliferative activity). Freezing media included the permeating cryoprotectant Me_2SO, either alone or along with the non-permeating cryoprotectant sucrose at 0.1 or 0.2 M. When Me_2SO was the only cryoprotectant, survival rate fell in thawed foetal cells to $54 \pm 4\%$ (against $89 \pm 6\%$ for thawed adult ones) and both proliferative and metabolic activities remained significantly lower than values for thawed adult cells. However, the combination of sucrose (both 0.1 as 0.2) and Me_2SO in foetal cells significantly increased their survival rates (to $71 \pm 4\%$ and $73 \pm 5\%$, respectively), proliferative activities (partially at day 7 and completely at day 14 after thawing) and metabolic activities.

Our findings clearly show a difference between foetal and adult cells concerning their cryopreservation sensitivity and requirements, as well as their recovery time after thawing. These results are of relevance for the cryopreservation of foetal and adult cells from the Iberian lynx and could be also useful for other mammals.

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Introduction

Advances in cell biotechnology increase the interest on the creation of cell banks, in order to be used as sources of different cell types when they are needed. This implies a parallel study concerning the knowledge of cell-type specific cryopreservation. Cryobanking of cells and tissues is an important and useful approach both for human applications [2,9,10] and animal preservation [4,7,15,16,18,22]. 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 for animal genetic preservation and conservation management, especially for threatened and endangered species. Critically endangered species are vulnerable to catastrophic events such as epizooties [24], which has resulted in increasing the interest on techniques for maintaining their genetic diversity [14]. Consequently, in a context of conservation biology, biomaterials from endangered species are being preserved for Spain's endangered wildlife [14–17], Southern Africa's wildlife [5], the Frozen Zoo of San Diego [25,26] or the Frozen Ark Consortium (http://www.frozenark.org), among others.

The Iberian lynx (*Lynx pardinus*) is considered the most endangered felid in the world [12]. Despite the increase in the number of individuals in the last years, Iberian lynxes are confined to two isolated populations in southern Spain, in the Doñana-Aljarafe and Sierra Morena areas, and only 88 and 224 individuals respectively are estimated to remain [28]. To protect this species from extinction, an EU LIFE Nature project is underway, which includes habitat preservation, lynx population monitoring, and rabbit population

☆ *Statement of funding:* This work was financed through projects from the Consejería de Medio Ambiente de la Junta de Andalucía-Universidad Miguel Hernández (CONSEMEDAMAND1.05X), BanCaja-Universidad Miguel Hernández (BANCAJA-UMH.03X) and Diputación de Alicante (DIPU2.04X).

* Corresponding author. Fax: +34 965222033.
E-mail address: trini.leon@umh.es (T. León-Quinto).

http://dx.doi.org/10.1016/j.cryobiol.2014.02.001
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management [27]. Additionally, a biological resource bank [15,16] as well as a captive *ex situ* breeding project [34] were initiated to preserve the genetic diversity of the species and to produce new specimens for future reintroduction. Biological resource banks include several types of cells and tissues, blood and derivatives, urine, pulled hairs or feces. Such biomaterials provide samples to future reproductive opportunities as well as to perform any study directed towards the conservation of the species [15–17,1,3].

Despite relevant improvement of gamete cryopreservation is being carried out in endangered species [32], intensive study of the reproductive biology of wild animals is still required [11]. Collection of gametes is often difficult, especially from females [15], so the banking of a genetic pool representative of the population biodiversity is very difficult when only gametes are considered. Cryobanking of somatic tissues and cells allows instead the preservation of the largest genetic biodiversity as well as to dispose of the samples needed when appropriate techniques will be fully developed [16].

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. Production of iPS cells from foetal fibroblasts was firstly reported in mouse [31]. Concerning highly endangered species, pluripotency has also been induced in somatic cells from a primate, the drill (*Mandrillus leucophaeus*), from the nearly extinct white rhinoceros (*Ceratotherium simum cottoni*) [6], as well as from the snow leopard (*Panthera uncial*) [35]. 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 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 [29,30]. Indeed, nuclear transfer using embryonic stem cells was more efficient than when done with differentiated cells in mouse and bovine. In the same way, foetal fibroblasts have been used in SCNT as donor nuclei since they are less differentiated than adult cells [13,36]. Even if SCNT has obvious potential and protocols are being continuously improved [4], its wide application is prevented by the still low efficiency in terms of offspring outcomes [2]. However, properly cryobanking of somatic cells in order to implement somatic cell banks for future use, when the procedure of SCNT and other biotechnologies have improved, should be considered an useful tool in conservation programs of threatened animal species [15,16,4,2].

Somatic foetal cells from endangered species are then a valuable biomaterial. They could be later used to reintroduce the lost genes into the breeding pool by means of different biotechnology techniques. The development of protocols to properly cryopreserve somatic foetal cells is therefore convenient for conservation programs. In a recent previous work [16], our group delimited for the first time the optimal culture and freezing conditions for adult tissues and cells from Iberian lynx skin biopsies. In such a previous work, we performed a complete and systematic study by covering a broad range of possibilities, analyzing twenty different culture conditions as well as fifteen different freezing solutions. Our findings revealed that the permeating cryoprotectant dimethyl sulfoxide (Me_2SO) at 10% v/v, resulted to be optimal for the cryopreservation of Iberian lynx adult fibroblasts. Likewise, addition of sucrose at 0.1 or 0.2 M to the freezing medium containing 10% Me_2SO, did not cause any improvement in the behavior of such cells after thawing.

Cryopreservation requirements however are not always the same for foetal somatic cells as for adult ones, because of their different response to cryoinjury at the same conditions. In this sense, when adult and foetal bovine somatic cells were cryopreserved in 10% Me_2SO [7], foetal cells exhibited after thawing viabilities significantly smaller than those observed in adult ones. Otherwise, the number of intact thawed human foetal cells also dropped to 44% when human foetal skin was previously cryopreserved with Me_2SO at 10% [9]. This percentage increased when a disaccharide as trehalose was added to the cryopreservation medium containing 10% Me_2SO, and the number of thawed human foetal cells presenting membrane integrity rose to 65%.

Taking into account the potential relevance of foetal somatic cells in order to later try to obtain both gametes and individuals, as well as a possible specificity concerning cryopreservation requirements, our global goal was to establish optimal freezing procedures related to foetal skin cells. For this purpose, the present study aimed at evaluating for the first time the ability of foetal cells from the Iberian lynx to be cryopreserved, in comparison with completely differentiated adult cells. We analyzed the impact of several cryopreservation media on both the cell viability (by analyzing their membrane integrity) and the cell functionality (by measuring their metabolic activity, glycosaminoglycan content and proliferative activity) of thawed foetal and adult skin cells. Responses were compared between both thawed cell types taking into account values from their respective fresh unfrozen controls. The freezing media analyzed contained the permeating cryoprotectant Me_2SO at 10% v/v, either alone or combined along with the non-permeating cryoprotectant sucrose at 0.1 or 0.2 M.

Materials and methods

Skin sampling

The collection of skin samples was performed under agreement with the Environmental Council of the Regional Government of Andalusia. We implemented protocols to collect and send samples from living and dead animals and provided sampling kits with the appropriate media [15]. 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 from living adult animals consisted of millimeter-sized skin biopsies, which were isolated always profiting from animal manipulation for other reasons (radio tracking, sanitary check-ups, etc.). Skin foetal samples were taken from recently dead fetuses from spontaneous abortions or dead pregnant females.

Skin samples were taken from fifteen healthy adult animals and ten fetuses by veterinarians from the Iberian lynx Conservation 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 Epidermal Growth Factor (EGF), 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 penicillin and 0.1 mg/mL streptomycin and 1% fungizone.

Culture medium

In this study we used the optimal culture medium and conditions previously investigated and described by us for adult cells from the Iberian lynx [16]. Briefly, the basal culture medium was

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