

УДК 57.043:599.742.75

CRYOPRESERVATION OF SKIN FIBROBLASTS OF THE SNOW LEOPARD (*PANTHERA UNCIA*) FROM KAZAKHSTAN: PASSIVE SLOW FREEZING VERSUS CONTROLLED-RATE FREEZING WITH DIMETHYL SULFOXIDE OR ETHYLENE GLYCOL

Yerzhan Toishibekov¹ , Alexey Grachev¹ , Madina Salmenova^{1*} , Tulendy Nurkenov¹ , Bibigul Katubayeva¹ ,
Dinmukhamed Toishybek¹ , Indira Kumayeva¹ , Maxim Beshpalov¹ , Sergey Beshpalov¹ , Saltore Saparbayev¹ 

Institute of Zoology, Committee of Science, Ministry of Science and Higher Education of the Republic of Kazakhstan, Almaty, Kazakhstan

*Corresponding author: Madina Salmenova, leleo@bk.ru

ABSTRACT

The snow leopard (*Panthera uncia*) is a Vulnerable felid, and cryobanking of its somatic cells is a practical way to preserve the genetic diversity of the Kazakhstan population. We compared the post-thaw viability of snow leopard skin fibroblasts after two slow-freezing protocols. Primary fibroblast lines were established from ear-skin biopsies of 16 animals and cryopreserved at passage 5. In experiment 1, cells were frozen passively in cryovials placed in a polystyrene block at -70°C (about $1^{\circ}\text{C}/\text{min}$) in fetal bovine serum with 10% dimethyl sulfoxide (DMSO). In experiment 2, cells were frozen in 0.5 mL straws in a programmable freezer with 1.5 M DMSO or 1.5 M ethylene glycol (EG). Viability was assessed by trypan blue exclusion. Passive freezing reduced viability from $98.5 \pm 0.3\%$ to $43.7 \pm 12.5\%$ ($p < 0.001$). Controlled-rate freezing yielded $57.9 \pm 9.4\%$ viable cells with DMSO and $62.9 \pm 8.9\%$ with EG; the difference between the two cryoprotectants was not significant ($p = 0.13$). Both controlled-rate variants outperformed passive freezing, by 14.2 and 19.2 percentage points (one-way ANOVA, $F(2,45) = 14.7$, $p < 0.001$; Tukey test, $p \leq 0.001$). Thawed cells reattached, proliferated and reached confluence within 7 days. Controlled-rate freezing in straws is recommended for the snow leopard somatic cell bank, and DMSO and EG can be used interchangeably. The resulting fibroblast collection forms the first somatic cell cryobank of snow leopards in Kazakhstan.

Keywords: snow leopard; *Panthera uncia*; fibroblasts; cryopreservation; controlled-rate freezing; dimethyl sulfoxide; ethylene glycol; cryobank.

1. INTRODUCTION

The snow leopard (*Panthera uncia* Schreber, 1775) inhabits the high mountains of Central and South Asia and is listed as Vulnerable on the IUCN Red List; its main threats are poaching, depletion of wild prey, conflict with livestock herders and habitat fragmentation [1]. In Kazakhstan the species is included in the national Red Book and occurs in the Tien Shan, Dzungarian Alatau, Saur-Tarbagatai and Altai ranges [2]. Populations of large felids are small, scattered and slow to recover, so every individual carries a noticeable share of the remaining genetic diversity.

Genome resource banks complement in situ protection by storing germplasm and somatic material at -196°C for future use [3–5]. Somatic cell banks are particularly practical for wild carnivores: a small skin biopsy can be taken from an animal of any sex and age during immobilisation, and fibroblast lines derived from it provide DNA for genomic studies, donor nuclei for somatic cell nuclear transfer and starting material for induced pluripotent stem cells [6, 7]. In felids this approach has been implemented for the Iberian lynx [8], the African wildcat, which was cloned from fibroblasts [9], the jaguar [10] and the Bengal tiger [11].

Reproductive and cellular technologies for the snow leopard remain poorly developed. Published work concerns mainly semen characteristics and sperm function in captive males [12, 13], and induced pluripotent stem cells were derived from snow leopard fibroblasts [14]. To our knowledge, however, freezing protocols for snow leopard somatic cells have not been compared, although the choice of protocol determines how much of a banked sample survives thawing.

Cell survival after slow freezing depends on the cooling rate and on the cryoprotectant. Cooling that is too fast leads to

intracellular ice, while cooling that is too slow prolongs exposure to concentrated solutes [15–17]. Passive freezing devices are cheap and simple, but they do not control the moment of ice nucleation or the cooling profile, which increases sample-to-sample variation [18]. Programmable freezers make the cooling reproducible. Dimethyl sulfoxide (DMSO) and ethylene glycol (EG) are the most widely used permeating cryoprotectants, and results obtained with them for felid somatic cells are contradictory: in jaguar cells EG was inferior to DMSO [19], whereas in puma cells the two agents gave similar results [20].

The aim of this study was to compare the post-thaw viability of snow leopard skin fibroblasts after passive slow freezing with DMSO and after controlled-rate freezing with DMSO or EG, and to choose a protocol for the somatic cell cryobank of snow leopards in Kazakhstan. The same laboratory has recently applied a similar comparison to saiga fibroblasts (Toishibekov et al., submitted).

2. MATERIALS AND METHODS

2.1. Animals and sampling

Skin samples were taken from the outer surface of the ear of 16 wild-caught snow leopards. The biopsy site was treated with 70% ethanol, and samples were placed in containers with transport medium (DMEM with 10% fetal bovine serum (FBS) and an antibiotic solution at 100 IU/mL) and delivered to the laboratory at $20\text{--}24^{\circ}\text{C}$ within 72 h. Live capture, handling and biopsy sampling were approved by the institutional animal care and ethics committee of the Institute of Genetics and Physiology, Committee of Science, Ministry of Science and Higher Education of the Republic of Kazakhstan, Almaty, Kazakhstan (protocol No. 3, 30 October 2023).

2.2. Primary culture of fibroblasts

In a class II biological safety cabinet (ESCO), samples were washed twice in Dulbecco's phosphate-buffered saline (DPBS; Sigma, USA) and cut into pieces of about 1 mm³ in a small volume of growth medium. Explants were placed on the bottom of 25 cm² culture flasks and covered with DMEM (Sigma, USA) supplemented with FBS 10 %, L-glutamine and an antibiotic solution (all Sigma, USA). Cultures were kept at 37 °C in a humidified atmosphere (85%) with 5% CO₂. Each animal gave one independent cell line. Cells were passaged at confluence by trypsinisation, and cells of passage 5 were used for cryopreservation. Only reagents from Sigma (USA) were used, and aseptic procedures followed the recommendations of the International Embryo Technology Society.

2.3. Experiment 1: passive slow freezing

The cell suspension was centrifuged at 160 g for 10 min, and the pellet was resuspended in FBS containing 10% DMSO (Sigma, USA) at $1-10 \times 10^6$ cells/mL. The suspension was transferred to cryovials, which were placed in the wells of a polystyrene foam block with 1 cm walls and put into a freezer at -70 °C; under these conditions the sample cooled at about 1 °C/min. After 24 h the cryovials were transferred to liquid nitrogen.

2.4. Experiment 2: controlled-rate freezing

Cells were frozen with 1.5 M DMSO or 1.5 M EG (Sigma, USA) prepared in DPBS, at a final concentration of 2×10^6 cells/mL. The suspension was loaded into 0.5 mL straws (CryoBioSystem, France), which were sealed with straw sealant (MiniTube, Germany). Straws were held at 5 °C for 2 h for equilibration and then cooled in a Planer Kryo 360-3.3 programmable freezer (Planer, UK) from 5 °C to -40 °C at 1 °C/min and from -40 °C to -85 °C at 4 °C/min, after which they were plunged into liquid nitrogen. In both experiments the cells of each line were divided among the treatments, so that every line was represented in every group (n = 16 per group).

2.5. Thawing and viability assessment

Cryovials were kept at room temperature for a few seconds to let residual nitrogen evaporate and then placed in a water bath at 37 °C; straws were thawed in a water bath at 37 °C for 5 min. The cryoprotectant was removed by dilution in a 20-fold volume of DMEM and centrifugation at 160 g for 10 min, and cells were resuspended in complete DMEM with 10% FBS, 2 mM L-glutamine and antibiotics [21]. The cell suspension was mixed with 0.4% trypan blue and counted in a Goryaev haemocytometer; unstained cells were scored as viable and stained cells as dead, and viability was expressed as the percentage of viable cells [22]. Viability of the same suspensions was determined before freezing. Part of the thawed cells was returned to culture in DMEM with 10% FBS to check their ability to attach and proliferate.

2.6. Cryobank

For long-term storage, streptomycin (50 µg/mL) was added to the cryoprotective medium. Cells and skin samples of all animals were stored in liquid nitrogen at -196 °C in the Germplasm Biobank of Wild Animals of the Institute of Zoology.

2.7. Statistical analysis

Viability is given as mean $\pm$ standard deviation (SD) with 95% confidence intervals (CI); n = 16 cell lines per group. Viability before and after freezing (experiment 1) and the two cryoprotectants (experiment 2) were compared with Welch's t-test. The three freezing variants were compared by one-way ANOVA followed by Tukey's test. Differences were considered significant at $p < 0.05$. Calculations were made from group means, SD and n = 16 (Python 3.11).

3. RESULTS

3.1. Primary cultures

Fibroblast lines were obtained from all 16 animals. Cultures consisted of spindle-shaped cells typical of fibroblasts. Viability of cells at passage 5 before freezing was $98.5 \pm 0.3\%$.

3.2. Experiment 1: passive slow freezing

After passive freezing with 10% DMSO, $43.7 \pm 12.5\%$ of cells were viable, i.e. viability fell by 54.8 percentage points (Table 1). The spread between lines was wide (coefficient of variation 29%). Thawed cells cultured in DMEM with 10% FBS attached and formed a confluent monolayer within 7 days, after which they were passaged. The cultures had the appearance of typical fibroblasts without epithelial cells or visible debris.

3.3. Experiment 2: controlled-rate freezing with DMSO or EG

After controlled-rate freezing, viability was $57.9 \pm 9.4\%$ with 1.5 M DMSO and $62.9 \pm 8.9\%$ with 1.5 M EG (Table 2). EG gave on average 5.0 percentage points more viable cells, but the difference was not significant ($p = 0.13$), and its 95% CI included zero.

3.4. Comparison of freezing variants

The freezing variant had a significant effect on post-thaw viability (one-way ANOVA, $F(2,45) = 14.7$, $p < 0.001$; Figure 1). Controlled-rate freezing with DMSO gave 14.2 percentage points more viable cells than passive freezing (95% CI 5.3–23.1; Tukey test, $p = 0.001$), and controlled-rate freezing with EG gave 19.2 percentage points more (95% CI 10.3–28.1; $p < 0.001$). The two controlled-rate variants did not differ ($p = 0.37$). Relative to viability before freezing, 44% of cells survived passive freezing, 59% survived controlled-rate freezing with DMSO and 64% with EG.

Table 1. Viability of snow leopard skin fibroblasts before and after passive slow freezing with 10% DMSO (n = 16)

Stage	n	Viable cells, % (M $\pm$ SD)	95% CI
Before freezing	16	98.5 ± 0.3	98.3–98.7
After thawing	16	43.7 ± 12.5	37.0–50.4
Difference		54.8	48.1–61.5

Welch's t-test: $t = 17.5$, $p < 0.001$.

Table 2. Post-thaw viability of snow leopard skin fibroblasts after controlled-rate freezing with 1.5 M DMSO or 1.5 M ethylene glycol (EG) (n = 16 per group; viability before freezing $98.5 \pm 0.3\%$)

Cryoprotectant	n	Viable cells, % (M $\pm$ SD)	95% CI
1.5 M DMSO	16	57.9 ± 9.4	52.9–62.9
1.5 M EG	16	62.9 ± 8.9	58.2–67.6
Difference (EG – DMSO)		5.0	–1.6 to 11.6

Welch's t-test: $t = 1.55$, $df = 29.9$, $p = 0.13$ (not significant).

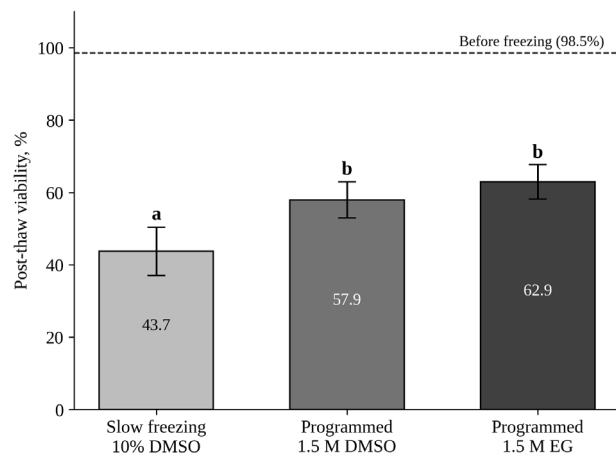


Figure 1. Post-thaw viability of snow leopard skin fibroblasts after passive slow freezing with 10% DMSO and controlled-rate freezing with 1.5 M DMSO or 1.5 M ethylene glycol (EG). Bars show means, whiskers 95% CI; n = 16 per group. Different letters indicate significant differences (one-way ANOVA, Tukey test, $p < 0.05$). The dashed line shows viability before freezing.

4. DISCUSSION

This study provides, to our knowledge, the first comparison of freezing protocols for snow leopard fibroblasts. The main result is that controlled-rate freezing in straws preserved 14–19 percentage points more viable cells than passive freezing in cryovials, while the choice between DMSO and EG at 1.5 M did not significantly affect the outcome.

The advantage of the programmable freezer is consistent with the basic principles of slow freezing. Survival is highest at a cooling rate that removes enough water from the cell to prevent intracellular ice but does not expose the cell to concentrated solutes for too long [15, 16]. In a passive device the actual cooling rate depends on the sample volume, the container and the load of the freezer, and ice nucleation occurs spontaneously after variable supercooling; the release of latent heat then raises the sample temperature and changes the cooling profile [17, 18]. These uncontrolled events are a likely cause of both the lower mean viability and the wider spread of results after passive freezing (29% coefficient of variation versus 16% and 14% in experiment 2).

Post-thaw viability of 44% after passive freezing is close to the values reported for other wild felids. Jaguar fibroblasts frozen with EG retained 45.8% viable cells compared with 97.8% before freezing, whereas DMSO gave better results in that study [19]. In puma cells, in contrast, reducing the concentration of DMSO or EG or replacing one with the other did not change viability [20]. Our data agree with the latter: at equal molarity, EG was slightly but not significantly better

than DMSO. EG has a lower molecular weight and permeates cells faster than DMSO, which may reduce osmotic stress during loading and dilution [17], but in snow leopard fibroblasts this did not translate into a significant gain. Both cryoprotectants can therefore be used, and the choice can be based on availability and on the intended downstream application.

The ability of thawed cells to attach and reach confluence within 7 days shows that the banked material remains suitable for further culture. Snow leopard fibroblasts have already been reprogrammed into induced pluripotent stem cells [14], and fibroblasts of other felids have served as donor cells for nuclear transfer [9]. A somatic cell bank covering many individuals of the Kazakhstan population is thus a realistic reserve for genetic studies and for future reproductive technologies [6, 7, 10].

The study has limitations. The two experiments differed not only in the cooling method but also in the container (cryovial versus straw), the carrier medium (FBS versus DPBS) and the cell concentration, so the effect of the cooling method cannot be fully separated from these factors; the DMSO concentration, however, was similar (10% DMSO corresponds to about 1.4 M). Viability was assessed only by trypan blue exclusion, which reflects membrane integrity but not metabolic activity, apoptosis or proliferation rate, which are affected by cryopreservation in felid cells [23–25]. Statistics were calculated from summary data with tests for independent groups; because every line was represented in every group, a paired analysis of the raw data would be more powerful.

5. CONCLUSION

Passive slow freezing of snow leopard skin fibroblasts with 10% DMSO preserved 43.7% viable cells, whereas controlled-rate freezing in 0.5 mL straws preserved 57.9% with 1.5 M DMSO and 62.9% with 1.5 M EG. Controlled-rate freezing was significantly better than passive freezing, and the two cryoprotectants did not differ significantly. Controlled-rate freezing in straws with 1.5 M EG or 1.5 M DMSO is recommended for the somatic cell cryobank of the snow leopard. Fibroblast lines of 16 snow leopards are now stored in the Germplasm Biobank of Wild Animals of the Institute of Zoology.

AUTHOR CONTRIBUTIONS

Yerzhan Toishibekov: Conceptualization, Methodology, Supervision, Project administration, Funding acquisition, Writing – review & editing. Alexey Grachev: Investigation, Resources. Madina Salmenova: Investigation, Formal analysis, Visualization, Writing – original draft. Tulendy Nurkenov, Bibigul Katubayeva, Dinmukhamed Toishybek, Indira Ku-

mayeva: Investigation. Maxim Beshpalov, Sergey Beshpalov, Saltore Saparbayev: Investigation, Resources. All authors read and approved the final manuscript.

FUNDING

This research was funded by the Science Committee of the Ministry of Science and Higher Education of the Republic of Kazakhstan (Grant No. AP23488891). Project title: “Studying the fundamental aspects of interspecies somatic cell nuclear transfer of snow leopard (*Panthera uncia*) for conservation of biological diversity”.

Conflict of interest. The authors declare no conflict of interest.

LITERATURE

- McCarthy T., Mallon D., Jackson R. et al. *Panthera uncia* // The IUCN Red List of Threatened Species 2017: e.T22732A50664030. – 2017. – DOI: 10.2305/IUCN.UK.2017-2.RLTS.T22732A50664030.en
- Krasnaya kniga Respubliki Kazakhstan [Red Book of the Republic of Kazakhstan]. – 4th ed. – Vol. 1: Animals. Part 1: Vertebrates. – Almaty: DPS, 2010. – 324 p. (In Russ.)
- Wildt D.E. Genome resource banking for wildlife research, management, and conservation // *ILAR Journal*. – 2000. – Vol. 41, № 4. – P. 228–234. – PMID: 11123183. – DOI: 10.1093/ilar.41.4.228
- Pukazhenth B., Comizzoli P., Travis A.J. et al. Applications of emerging technologies to the study and conservation of threatened and endangered species // *Reproduction, Fertility and Development*. – 2006. – Vol. 18, № 1–2. – P. 77–90. – PMID: 16478605. – DOI: 10.1071/rd05117
- Comizzoli P., Holt W.V. Breakthroughs and new horizons in reproductive biology of rare and endangered animal species // *Biology of Reproduction*. – 2019. – Vol. 101, № 3. – P. 514–525. – PMID: 30772911. – DOI: 10.1093/biolre/iox031
- Ryder O.A., Onuma M. Viable cell culture banking for biodiversity characterization and conservation // *Annual Review of Animal Biosciences*. – 2018. – Vol. 6. – P. 83–98. – PMID: 29447472. – DOI: 10.1146/annurev-animal-030117-014556
- Praxedes É.A., Borges A.A., Santos M.V.O. et al. Use of somatic cell banks in the conservation of wild felids // *Zoo Biology*. – 2018. – Vol. 37, № 4. – P. 258–263. – PMID: 29722907. – DOI: 10.1002/zoo.21416
- Leon-Quinto T., Simon M.A., Cadenas R. et al. Developing biological resource banks as a supporting tool for wildlife reproduction and conservation: the Iberian lynx bank as a model for other endangered species // *Animal Reproduction Science*. – 2009. – Vol. 112, № 3–4. – P. 347–361. – PMID: 18585877. – DOI: 10.1016/j.anireprosci.2008.05.070
- Gómez M.C., Pope C.E., Giraldo A. et al. Birth of African Wildcat cloned kittens born from domestic cats // *Cloning and Stem Cells*. – 2004. – Vol. 6, № 3. – P. 247–258. – PMID: 15671671. – DOI: 10.1089/clo.2004.6.247
- Mestre-Citrinovitz A.C., Sestelo A.J., Ceballos M.B. et al. Isolation of primary fibroblast culture from wildlife: the *Panthera onca* case to preserve a South American endangered species // *Current Protocols in Molecular Biology*. – 2016. – Vol. 116. – P. 28.7.1–28.7.14. – PMID: 27723084. – DOI: 10.1002/cpmb.25
- Guan W.J., Liu C.Q., Li C.Y. et al. Establishment and cryopreservation of a fibroblast cell line derived from Bengal tiger (*Panthera tigris tigris*) // *CryoLetters*. – 2010. – Vol. 31, № 2. – P. 130–138. – PMID: 20687455
- Roth T.L., Howard J.G., Donoghue A.M. et al. Function and culture requirements of snow leopard (*Panthera uncia*) spermatozoa in vitro // *Journal of Reproduction and Fertility*. – 1994. – Vol. 101, № 3. – P. 563–569. – PMID: 7966009. – DOI: 10.1530/jrf.0.1010563
- Johnston L.A., Armstrong D.L., Brown J.L. Seasonal effects on seminal and endocrine traits in the captive snow leopard (*Panthera uncia*) // *Journal of Reproduction and Fertility*. – 1994. – Vol. 102, № 1. – P. 229–236. – PMID: 7799318. – DOI: 10.1530/jrf.0.1020229
- Verma R., Holland M.K., Temple-Smith P. et al. Inducing pluripotency in somatic cells from the snow leopard (*Panthera uncia*), an endangered felid // *Theriogenology*. – 2012. – Vol. 77, № 1. – P. 220–228. – PMID: 22079579. – DOI: 10.1016/j.theriogenology.2011.09.022
- Mazur P., Leibo S.P., Chu E.H. A two-factor hypothesis of freezing injury. Evidence from Chinese hamster tissue-culture cells // *Experimental Cell Research*. – 1972. – Vol. 71, № 2. – P. 345–355. – PMID: 5045639. – DOI: 10.1016/0014-4827(72)90303-5
- Mazur P. Freezing of living cells: mechanisms and implications // *American Journal of Physiology*. – 1984. – Vol. 247, № 3, Pt 1. – P. C125–C142. – PMID: 6383068. – DOI: 10.1152/ajpcell.1984.247.3.C125
- Pegg D.E. Principles of cryopreservation // *Methods in Molecular Biology*. – 2007. – Vol. 368. – P. 39–57. – PMID: 18080461. – DOI: 10.1007/978-1-59745-362-2_3
- Morris G.J., Acton E. Controlled ice nucleation in cryopreservation – a review // *Cryobiology*. – 2013. – Vol. 66, № 2. – P. 85–92. – PMID: 23246475. – DOI: 10.1016/j.cryobiol.2012.11.007
- Oliveira L.R.M., Praxedes É.A., Silva M.B. et al. Comparative effect of cryoprotectant combinations on the conservation of somatic cells derived from jaguar, *Panthera onca*, towards the formation of biologic banks // *Anais da Academia Brasileira de Ciências*. – 2021. – Vol. 93, № 4. – Article e20190314. – PMID: 34705929. – DOI: 10.1590/0001-3765202120190314
- Rodrigues L.L.V., Moura Y.B.F., Viana J.V.S. et al. Comparison between concentration and type of intracellular cryoprotectants and the presence of sucrose for cryobanks of somatic cells derived from captive pumas // *Zoo Biology*. – 2023. – Vol. 42, № 3. – P. 440–448. – PMID: 36515220. – DOI: 10.1002/zoo.21748
- Freshney R.I. *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications*. – 7th ed. – Hoboken, NJ: Wiley-Blackwell, 2016. – 728 p.
- Strober W. Trypan blue exclusion test of cell viability // *Current Protocols in Immunology*. – 2015. – Vol. 111. – P. A3.B.1–A3.B.3. – PMID: 26529666. – DOI: 10.1002/0471142735.ima03bs111

23. Silva M.B., Praxedes É.A., Borges A.A. et al. Evaluation of the damage caused by in vitro culture and cryopreservation to dermal fibroblasts derived from jaguars: an approach to conservation through biobanks // *Zoo Biology*. – 2021. – Vol. 40, № 4. – P. 288–296. – PMID: 33755259. – DOI: 10.1002/zoo.21606

24. Borges A.A., Lira G.P.O., Nascimento L.E. et al. Isolation, characterization, and cryopreservation of collared peccary skin-derived fibroblast cell lines // *PeerJ*. – 2020. – Vol. 8. – Article e9136. – PMID: 32547858. – DOI: 10.7717/peerj.9136

25. Praxedes É.A., Oliveira L.R.M., Silva M.B. et al. Effects of cryopreservation techniques on the preservation of ear skin – an alternative approach to conservation of jaguar, *Panthera onca* (Linnaeus, 1758) // *Cryobiology*. – 2019. – Vol. 88. – P. 15–22. – PMID: 31029688. – DOI: 10.1016/j.cryobiol.2019.04.007

REFERENCES

1. McCarthy T., Mallon D., Jackson R. et al. *Panthera uncia* // The IUCN Red List of Threatened Species 2017: e.T22732A50664030. – 2017. – DOI: 10.2305/IUCN.UK.2017-2.RLTS.T22732A50664030.en

2. Krasnaya kniga Respubliki Kazakhstan [Red Book of the Republic of Kazakhstan]. – 4th ed. – Vol. 1: Animals. Part 1: Vertebrates. – Almaty: DPS, 2010. – 324 p. (In Russ.)

3. Wildt D.E. Genome resource banking for wildlife research, management, and conservation // *ILAR Journal*. – 2000. – Vol. 41, № 4. – P. 228–234. – PMID: 11123183. – DOI: 10.1093/ilar.41.4.228

4. Pukazhenthi B., Comizzoli P., Travis A.J. et al. Applications of emerging technologies to the study and conservation of threatened and endangered species // *Reproduction, Fertility and Development*. – 2006. – Vol. 18, № 1–2. – P. 77–90. – PMID: 16478605. – DOI: 10.1071/rd05117

5. Comizzoli P., Holt W.V. Breakthroughs and new horizons in reproductive biology of rare and endangered animal species // *Biology of Reproduction*. – 2019. – Vol. 101, № 3. – P. 514–525. – PMID: 30772911. – DOI: 10.1093/biolre/iz031

6. Ryder O.A., Onuma M. Viable cell culture banking for biodiversity characterization and conservation // *Annual Review of Animal Biosciences*. – 2018. – Vol. 6. – P. 83–98. – PMID: 29447472. – DOI: 10.1146/annurev-animal-030117-014556

7. Praxedes É.A., Borges A.A., Santos M.V.O. et al. Use of somatic cell banks in the conservation of wild felids // *Zoo Biology*. – 2018. – Vol. 37, № 4. – P. 258–263. – PMID: 29722907. – DOI: 10.1002/zoo.21416

8. Leon-Quinto T., Simon M.A., Cadenas R. et al. Developing biological resource banks as a supporting tool for wildlife reproduction and conservation: the Iberian lynx bank as a model for other endangered species // *Animal Reproduction Science*. – 2009. – Vol. 112, № 3–4. – P. 347–361. – PMID: 18585877. – DOI: 10.1016/j.anireprosci.2008.05.070

9. Gómez M.C., Pope C.E., Giraldo A. et al. Birth of African Wildcat cloned kittens born from domestic cats // *Cloning and Stem Cells*. – 2004. – Vol. 6, № 3. – P. 247–258. – PMID:

15671671. – DOI: 10.1089/clo.2004.6.247

10. Mestre-Citrinovit A.C., Sestelo A.J., Ceballos M.B. et al. Isolation of primary fibroblast culture from wildlife: the *Panthera onca* case to preserve a South American endangered species // *Current Protocols in Molecular Biology*. – 2016. – Vol. 116. – P. 28.7.1–28.7.14. – PMID: 27723084. – DOI: 10.1002/cpmb.25

11. Guan W.J., Liu C.Q., Li C.Y. et al. Establishment and cryopreservation of a fibroblast cell line derived from Bengal tiger (*Panthera tigris tigris*) // *CryoLetters*. – 2010. – Vol. 31, № 2. – P. 130–138. – PMID: 20687455

12. Roth T.L., Howard J.G., Donoghue A.M. et al. Function and culture requirements of snow leopard (*Panthera uncia*) spermatozoa in vitro // *Journal of Reproduction and Fertility*. – 1994. – Vol. 101, № 3. – P. 563–569. – PMID: 7966009. – DOI: 10.1530/jrf.0.1010563

13. Johnston L.A., Armstrong D.L., Brown J.L. Seasonal effects on seminal and endocrine traits in the captive snow leopard (*Panthera uncia*) // *Journal of Reproduction and Fertility*. – 1994. – Vol. 102, № 1. – P. 229–236. – PMID: 7799318. – DOI: 10.1530/jrf.0.1020229

14. Verma R., Holland M.K., Temple-Smith P. et al. Inducing pluripotency in somatic cells from the snow leopard (*Panthera uncia*), an endangered felid // *Theriogenology*. – 2012. – Vol. 77, № 1. – P. 220–228. – PMID: 22079579. – DOI: 10.1016/j.theriogenology.2011.09.022

15. Mazur P., Leibo S.P., Chu E.H. A two-factor hypothesis of freezing injury. Evidence from Chinese hamster tissue-culture cells // *Experimental Cell Research*. – 1972. – Vol. 71, № 2. – P. 345–355. – PMID: 5045639. – DOI: 10.1016/0014-4827(72)90303-5

16. Mazur P. Freezing of living cells: mechanisms and implications // *American Journal of Physiology*. – 1984. – Vol. 247, № 3, Pt 1. – P. C125–C142. – PMID: 6383068. – DOI: 10.1152/ajpcell.1984.247.3.C125

17. Pegg D.E. Principles of cryopreservation // *Methods in Molecular Biology*. – 2007. – Vol. 368. – P. 39–57. – PMID: 18080461. – DOI: 10.1007/978-1-59745-362-2_3

18. Morris G.J., Acton E. Controlled ice nucleation in cryopreservation – a review // *Cryobiology*. – 2013. – Vol. 66, № 2. – P. 85–92. – PMID: 23246475. – DOI: 10.1016/j.cryobiol.2012.11.007

19. Oliveira L.R.M., Praxedes É.A., Silva M.B. et al. Comparative effect of cryoprotectant combinations on the conservation of somatic cells derived from jaguar, *Panthera onca*, towards the formation of biologic banks // *Anais da Academia Brasileira de Ciências*. – 2021. – Vol. 93, № 4. – Article e20190314. – PMID: 34705929. – DOI: 10.1590/0001-376520210190314

20. Rodrigues L.L.V., Moura Y.B.F., Viana J.V.S. et al. Comparison between concentration and type of intracellular cryoprotectants and the presence of sucrose for cryobanks of somatic cells derived from captive pumas // *Zoo Biology*. – 2023. – Vol. 42, № 3. – P. 440–448. – PMID: 36515220. – DOI: 10.1002/zoo.21748

21. Freshney R.I. *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications*. – 7th ed. – Hoboken, NJ: Wiley-Blackwell, 2016. – 728 p.

22. Strober W. Trypan blue exclusion test of cell viability // Current Protocols in Immunology. – 2015. – Vol. 111. – P. A3.B.1–A3.B.3. – PMID: 26529666. – DOI: 10.1002/0471142735.ima03bs111

23. Silva M.B., Praxedes É.A., Borges A.A. et al. Evaluation of the damage caused by in vitro culture and cryopreservation to dermal fibroblasts derived from jaguars: an approach to conservation through biobanks // Zoo Biology. – 2021. – Vol. 40, № 4. – P. 288–296. – PMID: 33755259. – DOI: 10.1002/zoo.21606

24. Borges A.A., Lira G.P.O., Nascimento L.E. et al. Iso-

lation, characterization, and cryopreservation of collared peccary skin-derived fibroblast cell lines // PeerJ. – 2020. – Vol. 8. – Article e9136. – PMID: 32547858. – DOI: 10.7717/peerj.9136

25. Praxedes É.A., Oliveira L.R.M., Silva M.B. et al. Effects of cryopreservation techniques on the preservation of ear skin – an alternative approach to conservation of jaguar, *Panthera onca* (Linnaeus, 1758) // Cryobiology. – 2019. – Vol. 88. – P. 15–22. – PMID: 31029688. – DOI: 10.1016/j.cryobiol.2019.04.007

КРИОКОНСЕРВАЦИЯ ФИБРОБЛАСТОВ КОЖИ СНЕЖНОГО БАРСА (*PANTHERA UNCIA*) ИЗ КАЗАХСТАНА: ПАССИВНОЕ МЕДЛЕННОЕ ЗАМОРАЖИВАНИЕ В СРАВНЕНИИ С ЗАМОРАЖИВАНИЕМ С КОНТРОЛИРУЕМОЙ СКОРОСТЬЮ В ПРИСУТСТВИИ ДИМЕТИЛСУЛЬФОКСИДА ИЛИ ЭТИЛЕНГЛИКОЛЯ

Ержан Тойшибеков¹, Алексей Грачев¹, Мадина Салменова^{1*}, Туленды Нуркенов¹, Бибигуль Катубаева¹, Динмухамед Тойшыбек¹, Индира Кумаева¹, Максим Беспалов¹, Сергей Беспалов¹, Салторе Сапарбаев¹

Институт зоологии Комитета науки Министерства науки и высшего образования Республики Казахстан, Алматы, Казахстан

**Автор корреспонденции: Мадина Салменова, leleo@bk.ru*

АННОТАЦИЯ

Снежный барс (*Panthera uncia*) относится к уязвимым видам кошачьих, и криобанк его соматических клеток — практический способ сохранить генетическое разнообразие казахстанской популяции. Мы сравнили жизнеспособность фибробластов кожи снежного барса после оттаивания при двух способах медленного замораживания. Первичные линии фибробластов получены из биоптатов кожи ушной раковины 16 животных и заморожены на 5-м пассаже. В опыте 1 клетки замораживали пассивно в криопробирках в пенополистирольном блоке при -70°C (около $1^{\circ}\text{C}/\text{мин}$) в фетальной бычьей сыворотке с 10% диметилсульфоксида (ДМСО). В опыте 2 клетки замораживали в соломинках 0,5 мл в программном замораживателе с 1,5 М ДМСО или 1,5 М этиленгликолем (ЭГ). Жизнеспособность оценивали по исключению трипанового синего. Пассивное замораживание снизило долю живых клеток с $98,5 \pm 0,3\%$ до $43,7 \pm 12,5\%$ ($p < 0,001$). При программном замораживании доля живых клеток составила $57,9 \pm 9,4\%$ с ДМСО и $62,9 \pm 8,9\%$ с ЭГ; различие между криопротекторами недостоверно ($p = 0,13$). Оба варианта программного замораживания превосходили пассивное на 14,2 и 19,2 процентного пункта (однофакторный дисперсионный анализ, $F(2,45) = 14,7$, $p < 0,001$; критерий Тьюки, $p \leq 0,001$). После оттаивания клетки прикреплялись, пролиферировали и достигали конfluence за 7 суток. Для криобанка соматических клеток снежного барса рекомендуется программное замораживание в соломинках; ДМСО и ЭГ взаимозаменяемы. Полученная коллекция фибробластов составляет первый криобанк соматических клеток снежного барса в Казахстане.

Ключевые слова: снежный барс; *Panthera uncia*; фибробласты; криоконсервация; программное замораживание; диметилсульфоксид; этиленгликоль; криобанк.

**ҚАЗАҚСТАННАН АЛЫНҒАН ҚАР БАРЫСЫНЫҢ (*PANTHERA UNCIA*) ТЕРІ
ФИБРОБЛАСТТАРЫН КРИОКОНСЕРВАЦИЯЛАУ: ДИМЕТИЛСУЛЬФОКСИД НЕМЕСЕ
ЭТИЛЕНГЛИКОЛЬ ҚАТЫСУЫМЕН ПАССИВТІ БАЯУ МҮЗДАТУ ЖӘНЕ БАҚЫЛАНАТЫН
ЖЫЛДАМДЫҚПЕН МҮЗДАТУ**

Ержан Тойшыбеков¹, Алексей Грачев¹, Мадина Салменова^{1*}, Туленды Нуркенов¹, Бибигүл Катубаева¹, Динмухамед Тойшыбек¹, Индира Кумаева¹, Максим Беспалов¹, Сергей Беспалов¹, Салторе Сапарбаев¹

Зоология институты, Ғылым комитеті, Қазақстан Республикасы Ғылым және жоғары білім министрлігі, Алматы, Қазақстан

**Тіліші автор: Мадина Салменова, leleo@bk.ru*

АНДАТПА

Ілбіс (*Panthera uncia*) — осал түрлер санатына жататын мысық тұқымдас жыртқыш, ал оның соматикалық жасушаларын криобанкте сақтау Қазақстан популяциясының генетикалық әртүрлілігін сақтаудың тиімді жолы болып табылады. Жұмыста ілбіс терісі фибробласттарының еріткеннен кейінгі тіршілікке қабілеттілігі баяу мұздатудың екі әдісі бойынша салыстырылды. Бастапқы фибробласт желілері 16 жануардың құлақ терісінің биоптаттарынан алынып, 5-пассада криоконсервацияланды. 1-тәжірибеде жасушалар 10% диметилсульфоксиді (ДМСО) бар фетальды бұзау сарысуында криопробиркаларда пенополистирол блокта -70°C температурада пассивті түрде (шамамен $1^{\circ}\text{C}/\text{мин}$) мұздатылды. 2-тәжірибеде жасушалар 0,5 мл соломинкаларда бағдарламалы мұздатқышта 1,5 М ДМСО немесе 1,5 М этиленгликольмен (ЭГ) мұздатылды. Тіршілікке қабілеттілік трипан көгімен бояу арқылы бағаланды. Пассивті мұздату тірі жасушалар үлесін $98,5 \pm 0,3\%$ -дан $43,7 \pm 12,5\%$ -ға дейін төмендетті ($p < 0,001$). Бағдарламалы мұздатуда тірі жасушалар ДМСО-мен $57,9 \pm 9,4\%$, ЭГ-мен $62,9 \pm 8,9\%$ құрады; криопротекторлар арасындағы айырмашылық сенімсіз ($p = 0,13$). Бағдарламалы мұздатудың екі нұсқасы да пассивті мұздатудан 14,2 және 19,2 пайыздық тармаққа жоғары болды (бірфакторлы дисперсиялық талдау, $F(2,45) = 14,7$, $p < 0,001$; Тьюки критерийі, $p \leq 0,001$). Еріткеннен кейін жасушалар бекініп, көбейіп, 7 тәулікте конfluence-ке жетті. Ілбістің соматикалық жасушалар банкі үшін соломинкаларда бағдарламалы мұздату ұсынылады; ДМСО мен ЭГ бір-бірін алмастыра алады. Алынған фибробласттар коллекциясы Қазақстандағы ілбістің алғашқы соматикалық жасушалар криобанкін құрайды.

Түйін сөздер: ілбіс; *Panthera uncia*; фибробласттар; криоконсервация; бағдарламалы мұздату; диметилсульфоксид; этиленгликоль; криобанк.