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Optimization of cryopreservation methods for somatic cells of the Tobet dog breed

Abstract. The Tobet dog breed, a national heritage of Kazakhstan, is threatened with extinction and conservation measures are urgently needed. This study presents a pioneering approach to preserve the genetic diversity and survival of this endangered breed through cryopreservation and cryobanking. We have optimised methods for cryopreservation of somatic cells (fibroblasts) isolated from skin explants of Tobet dogs and investigated the effects of in vitro cultivation on cell viability. Our results emphasise the importance of selecting appropriate culture media and cryoprotectants. Dulbecco's Modified Eagle Medium (DMEM) and ethylene glycol (EG) were found to be the most effective agents for increasing the growth rate and viability of fibroblasts after thawing. Through careful experimentation, including the evaluation of equilibrium and non-equilibrium cryopreservation techniques and the application of various cryoprotectants, we have established the first Tobet cryobank for somatic cells. The establishment of a cryobank represents a significant step towards the conservation of the Tobet dog breed and provides a model for the conservation of other endangered species. This study not only contributes to the preservation of Kazakhstan's cultural and biological heritage, but also opens up new avenues for the application of biotechnological approaches to wildlife conservation.

Key words: Tobet, cryopreservation, fibroblast, equilibrium programmable cryoconservation, non-equilibrium cryoconservation.

Introduction

Dogs of the national breed Tobet have been true friends and protectors of the Kazakh people for many centuries. But today they themselves need to be saved. Unfortunately, this unique breed is almost lost. Hundreds of years of work of our ancestors in breeding an ideal dog, a reliable guardian, a surprisingly strong and resistant dog can be in vain.

Currently, work to preserve, develop and restore the national Kazakh dog breeds, including the Tobet, is supported by the state. The success of this work will depend on civilized breeding work with them, which must be based on a scientific foundation. The modern arsenal of breeding methods has been significantly expanded by breakthrough achievements and new approaches in genetics, genomics, cytogenetics, and biotechnology.

Cryopreservation (i.e., freezing cells and tissues with cryoprotectants that ensure their long-term viable storage) followed by cryobanking (i.e., storing viable cells) may be a promising strategy to save an endangered breed. The effectiveness of cryobiological methods in conserving valuable livestock breeds and rare wildlife species has been demonstrated in some studies [1,2]. It has been shown that even a limited number of samples collected for long-term cryobanking can preserve genetic variation within a population. Today, cryobanks are used for the conservation and reproduction of such unique species as the giant panda (*Ailuropoda melanoleuca*), cheetah (Acinonyx jubatus), black-footed ferret (Mustela nigripe), Siberian crane (Leucogeranus leucogeranus), great bustard (*Chlamydotis undulata*), etc. [3-6] for supraregional IVF procedures of coral reefs [7]. However, little research has been conducted to develop procedures for cryopreservation of canine germplasm. Studies on sperm cryopreservation are more numerous [8-15]. In Kazakhstan, the successful cryopreservation of the sperm of a valuable producer of the Kazakh national breed Tazy is described [16]. Such artificial insemination with cryopreserved

sperm offers several advantages, including avoiding the stress associated with transporting the animal, solving the problem of reproduction associated with the behavior (aggressiveness or indifference) of the partner, and the quarantine imposed on the animal [11]. However, research on cryopreservation of canine somatic tissue, oocytes, and gonadal tissue is still insufficient [17-19], and is a priority in the field of canine germplasm cryobanking. It should be kept in mind that such cryobanking can only be successful if the protocol is optimized for specific species and possibly even breeds.

In this context, the aim of this study was to investigate the scientific basis for the long-term conservation of the somatic cells of the Kazakh national breed Tobet using various cryopreservation methods.

Materials and methods

The main object of the study were dogs of Tobet breed. The collection of biomaterials from Tobet dogs was carried out during expeditions, exhibitions, and special events. Testing for compliance with the breed characteristics of the Kazakh Tobet breed was carried out by cynologists from "KANSONAR", who met the qualification requirements and were experts in Kazakh national breeds and in the group such as the Central Asian Shepherd Dog (CAS), to which the Tobet belongs. The main document for conducting the test and establishing the breed standard that guided the experts was the breed standard for the Kazakh Tobet, which was approved by the Order of the Ministry of ecology and natural resources of the Republic of Kazakhstan dated 30 March 2023 No.101 "On the Approval of Standards for Kazakh Dog Breeds."

We used samples of skin explants from Tobet dogs. This type of biomaterial, obtained after ear cropping, was only collected from puppies of Tobet dogs. Ear cropping was performed by a professional veterinarian, was planned, and was only performed after the dog owner had signed a consent form for this procedure. The biomaterial was used for isolation and *in vitro* culture of somatic cells (fibroblasts) and is necessary for the subsequent establishment of a Tobet somatic cell cryobank.

The study was approved by the bioethics committee of the RSE at REM Institute of Molecular Biology and Biochemistry named after M.A.Aitkhozhin CS MSHE RK (Protocol No. 1, August 18, 2023). The study is based on the "Bioethical rules for conducting research on humans and animals" and is in accordance with the legislation of the Republic of Kazakhstan and the The European Convention on Bioethics. In the study, no experiments are conducted on the animals themselves, i.e., dogs; only biomaterials collected from dogs are used. The collection of all types of biomaterials mentioned in the study is a minimally invasive procedure that causes no harm to the dog.

In vitro culture of somatic cells (fibroblasts) isolated from skin explants. For the isolation and *in vitro* culture of somatic cells, fibroblasts, skin samples from the auricle of Tobet puppies were used during their docking (partial or complete removal of the external visible flap of the ear and/or ear cartilage of the animal), according to the external description of the breed, in a veterinary clinic by qualified specialists – veterinarians. Next, skin samples were treated with ethyl alcohol, placed in 15 ml plastic tubes with 10 ml of DMEM transport medium (Sigma, USA) containing 10% FBS and antibiotics at standard concentrations and transported to the laboratory.

In the laboratory, under the conditions of a sterile laminar flow hood (Esco, Singapore), skin samples were pre-rinsed twice in a DPBS solution (Sigma) for several minutes, the remaining hair was removed from their surface, then cut with a scalpel into pieces measuring approximately 1-2 mm^2 in a small volume of complete medium. After grinding, the tissue pieces were placed on the bottom of plastic culture mats with a surface area of 25 cm^2 and covered with complete culture medium in a volume of 3 ml, followed by observation of the release of fibroblasts from the explants and cell proliferation. For further *in vitro* culture of fibroblasts, DMEM nutrient medium (Sigma, USA) containing fetal bovine serum (Sigma, USA), L-glutamine (Sigma, USA) was used, and to suppress microflora, an antibiotic solution was added to the nutrient medium (Sigma, USA).

Further culturing of the explants was carried out in a CO_2 incubator (Binder, Germany), under conditions: 37°C, 5% CO_2 and 85% humidity for 4-5 days, without touching or moving the culture dishes. Using an inverted microscope Axiovert 40 CFL (Zeiss, Germany), the initial release and migration of single fibroblasts from the explant and the subsequent appearance of the growth zone were recorded. Cells were cultured in complete culture medium until they reached a monolayer in culture mats. To maintain the normal functioning of the cell culture, as well as to prevent negative phenomena, periodically, every 5 days, the culture medium was replaced with fresh one. To avoid contamination of cultures with bacteria

or other cell lines, all manipulations were carried out in compliance with aseptic rules in a sterile box Esco (Esco, Singapore).

Since fibroblasts are adhesive cells and form a monolayer in culture, for further passaging they must be separated from the substrate and their clusters separated. Cell dissociation and separation of the monolayer from the surface of the culture plastic was carried out using a warm 0.25% trypsin solution. The cells separated from the surface were collected into a 15-ml tube and washed by centrifugation. The resulting fibroblast pellet was resuspended in fresh culture medium, transferred to 2 new culture mats, and subsequent culturing was continued to increase the cell population, or the cells were frozen.

Study of the viability of somatic cells (fibroblasts) of the Tobet breed. Cell viability was assessed using the trypan blue dye exclusion method. The cell suspension was mixed with a 0.4% trypan blue solution and introduced into Goryaev's chamber. Separately stained (dead) and unstained (live) cells were counted under microscopy, thereby calculating the relative number (percentage) of living cells.

Slow freezing (Non-Equilibrium Cryopreservation, NEC) using various cryoprotectants (dimethyl sulfoxide and ethylene glycol). Fibroblasts were frozen using 0.5 ml straws (CryoBioSystem, France). For this purpose, the cell suspension was centrifuged for 10 min at 300 g. The cell sediment was resuspended in various cryoprotectants at the rate of 1-10 million cells per 1 ml of FTS, filled into 0.5 ml straws, which were placed for equilibration in a freezer chamber at -70°C. Then, after 24 hours, the straws with cells were transferred to a Dewar flask with liquid nitrogen. Various cryoprotectants were used for freezing: 10% dimethyl sulfoxide (DMSO) and 10% ethylene glycol (EG).

Slow program freezing (Equilibrium Programmable Cryoconservation, EPC) using a program freezer and various cryoprotectants (dimethyl sulfoxide and ethylene glycol). For equilibrium cryopreservation of cells, the slow freezing method was used using a Planer Kryo 330 – 3.3 software freezer (Planer, UK) (Figure 1). Fibroblasts were frozen in 2 different cryoprotectants: 1.5 M DMSO (Sigma, USA) and 1.5 M ethylene glycol (Sigma, USA), prepared in DMEM and 10% BSA. When diluting with a cryoprotector, a cell concentration of 2 x 106 cells/ml was maintained. For cryopreservation and further storage, 0.5 ml straws (CryoBioSystem, France) were filled with samples with a cryoprotector; the end of the straw was sealed. The samples were then placed in a refrigerator and kept for 2 hours at 5ºC. This procedure is necessary for equilibration and stabilization of cells in the cryoprotectant solution. Then they were frozen in a program freezer. For freezing, the following regime was used: from 5ºC to -40ºC at a rate of -1ºC/min, from -40ºC to -85ºC at a rate of -4ºC/min (Figure 1), and then the straws were transferred to liquid nitrogen for storage.

Thawing cells after freezing. Thawing of cells was carried out using a standard method (Freshney). The cryovial with cells was transferred from the Dewar flask to a water bath with a temperature of 37°C, having previously been kept at room temperature to evaporate the remaining liquid nitrogen from the test tube. After complete thawing, the cell suspension was washed from DMSO and fetal serum in a 20-fold excess of RPMI-1640 medium by centrifugation at 160 g for 10 min. Cells were then resuspended in complete RPMI-1640 culture medium containing 10% FCS, 2 mM L-glutamine, and antibiotics. After this, cell viability was determined by trypan blue exclusion and subjected to further cultivation.

Statistical data processing. For statistical processing, the ANOVA application program was used. We calculated the arithmetic mean, the mean square error of the arithmetic mean, and the reliability of the difference in means P using the Student's test.

Results and discussion

The study of the effects of *in vitro* cultivation of somatic cells on their viability is of strategic importance in the context of the conservation of valuable animal breeds, as it allows to develop optimal conditions for maintaining and ensuring high viability of their cells.

In this study, to isolate somatic cells, in particular fibroblasts, skin samples obtained from the auricle of a Tobet dog were used. The release of fibroblasts from explants and cell proliferation were monitored by microscopy. The rate of spreading and growth in culture dishes made of various materials (plastic and glass Petri dishes, plastic culture flasks) was assessed.

Observation of the release of cells from the explant tissue showed that, regardless of the method of explantation (in culture mats or in Petri dishes under a cover glass), the release of single cells from the explant tissue (Figure 1a) began no earlier than the 6th day of cultivation (on average 8-10th day after sowing) and was accompanied by cell division until a monolayer was reached (Figures 1b-d).

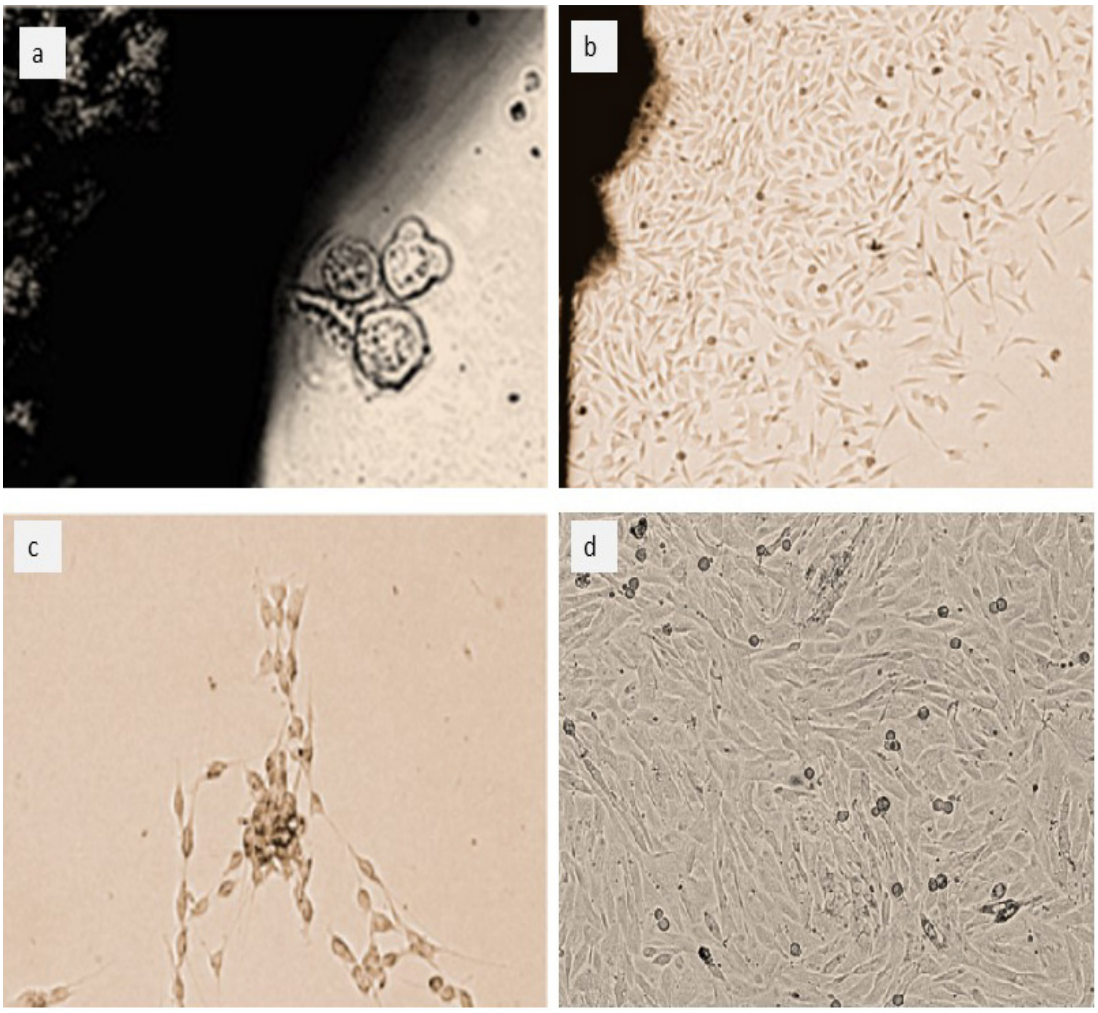

Figure 1 – Output of fibroblasts from explant tissue obtained from the skin of a Tobet dog.
Note: a – output of single fibroblasts from explant tissue obtained from the skin of a Tobet dog;
b – release and proliferation of fibroblasts from explant tissue obtained from skin on the 14th day of culturing (Tobet dog);
c – an island of dividing fibroblasts from the stroma of an explant obtained from the skin on the 14th day of culture (Tobet dog);
d – monolayer of fibroblasts from the stroma of the explant obtained from the skin on the 28th day of culture (Tobet dog)

Already 10-12 days after the fibroblasts emerged from the explant, the monolayer occupied approximately 1/3 of the surface of a culture mattress with an area of 25 cm^2 or a Petri dish with a diameter of 60 mm.

When comparing the effectiveness of the two culture media used (RPMI-1640 and DMEM), preference was given to DMEM. When cultured in this medium, fibroblasts emerged from the explant several days earlier, and their growth rate was also noticeably higher.

Passaging of fibroblasts was carried out to increase cell mass, as well as to obtain a monoculture. Since fibroblasts are adherent cells, proteolytic enzymes, the main of which is trypsin, are most often used to separate them from the surface of the culture dish. In our studies, the use of this enzyme alone turned out to be effective and sufficient for almost complete removal of the fibroblast monolayer from the surface of the culture dish. The optimal incubation time with trypsin was 7-10 minutes at 37°C. To prevent further action of the enzyme, 5-20 ml of 10% full complete media was added. The disaggregated cell suspension was transferred into new culture vessels, diluted 2-fold with fresh culture medium. Cell counting after trypsin dissociation showed that the percentage of living cells was 91.7 ± 0.6.

The dynamics of the process of desorption of fibroblasts from the surface of plastic Petri dishes is presented on Figure 2.

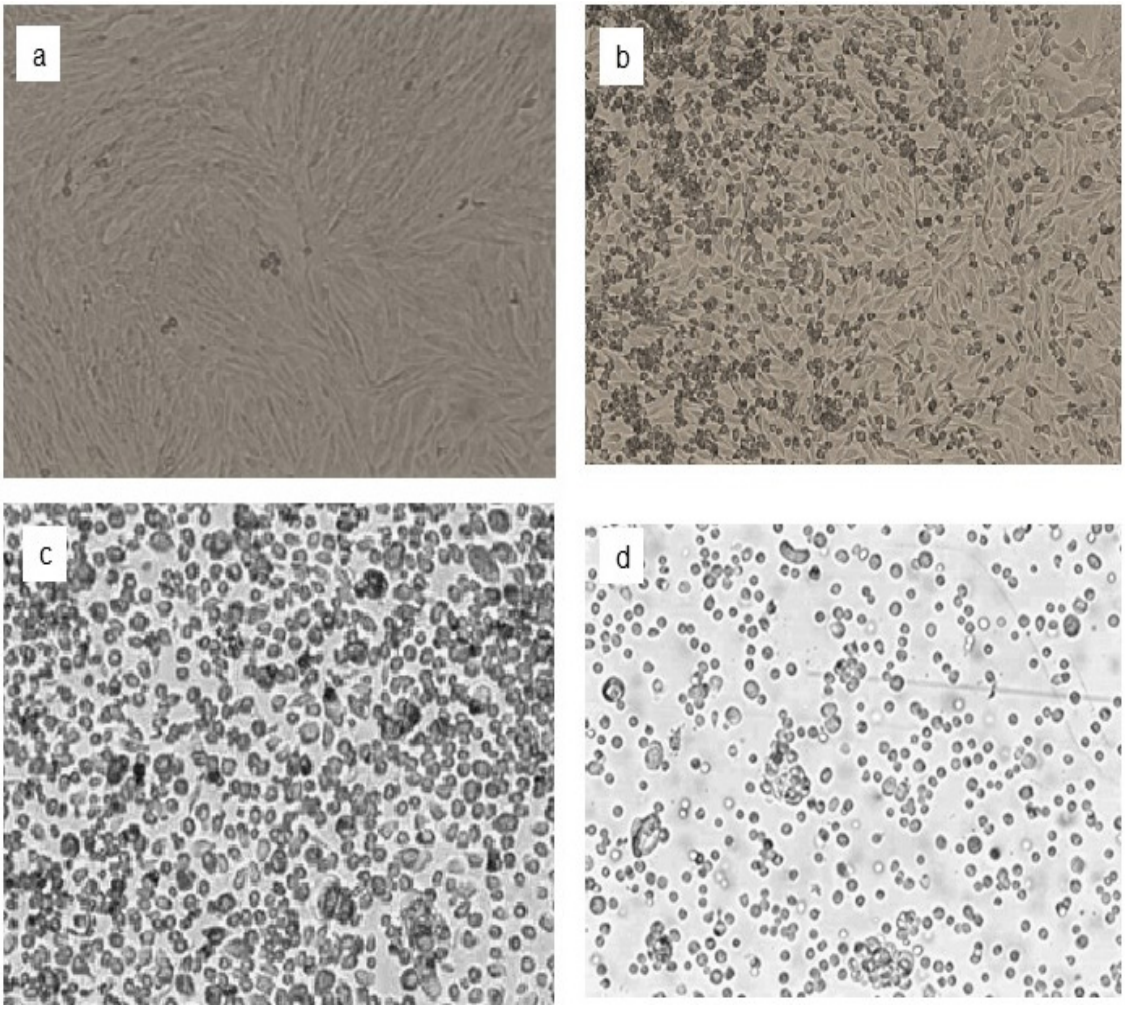

Figure 2 – The dynamics of the process of desorption of fibroblasts from the surface of plastic Petri dishes.
Note: a – monolayer of fibroblasts obtained from a skin explant after washing with a buffer solution;
b – culture of fibroblasts obtained from a skin explant 40 seconds after the addition of trypsin;
c – culture of fibroblasts obtained from a skin explant 5 minutes after the addition of trypsin;
d – culture of fibroblasts obtained from a skin explant 10 minutes after the addition of trypsin and after resuspension

After the next passage, carried out after the cell culture reached the confluency state, the cell growth rate increased significantly. Moreover, the cell culture became more homogeneous. So, after the 5th passage we received an almost pure monoculture.

Study the effect of slow freezing NEC using various cryoprotectants (dimethyl sulfoxide and ethylene glycol) on the viability of frozen-thawed fibroblasts of Tobet dogs. Slow freezing using cryoprotectants has been shown to be an effective method of preserving animal genetic material [2,20]. Despite the extensive studies on this method and the effective protocols available, the individual characteristics and response to cryopreservation procedures can vary considerably depending on the cell type and animal species or breed [21-27], making it necessary to individualise the approaches and optimise the protocols to produce viable cell lines.

As is known, the viability of cells during freezing is influenced by several factors, the main of which is the composition of the cryoprotectant and the freezing regime. The action of this factor is aimed at the formation of hexagonal ice crystals to preserve the integrity of the cytoplasmic membranes of cells. At the same time, it was revealed that there are species differences between cells in biophysical parameters: surface area, cell volume and membrane permeability of water. However, theoretical models have now been defined for different cell types, but these models do not correspond to fibroblasts (cell

size). Despite these differences, it is quite possible to use them to optimize the conditions for increasing the cryoviability of fibroblasts of various animal species and breeds.

The experiment used the simplest and most common method of cryopreservation. The results of assessing the viability rate of cells (fibroblasts) of Tobet dogs subjected to cryopreservation are presented in Table 1.

The viability rate of Tobet dog fibroblasts using various cryoprojectors: 10% DMSO and 10% EG, as well as the use of slow freezing NEC was 54.3 ± 1.1% and 65.7 ± 1.1 % respectively. Static analysis by Student did not reveal significant differences (P > 0.05). However, it should be noted that when using the 10% EG, the viability rate of frozen-thawed fibroblasts of Tobet dogs is higher than when using the 10% DMSO.

Table 1 – Effect of various cryoprotectants and slow freezing NEC on the viability of Tobet fibroblasts

Cryoprotector/freezing mode	Relative number of living cells before freezing, %	Relative number of living cells after thawing, %
DMSO/ NEC	91.7 ± 0.6a	54.3 ± 1.1b
EG/ NEC	91.7 ± 0.6a	65.7 ± 1.1c
Note, ab, bc P > 0.05		

Study the effect of slow program freezing EPC using a program freezer and various cryoprotectants (dimethyl sulfoxide and ethylene glycol) on the viability of frozen-thawed fibroblasts of Tobet dogs.

In contrast to the previous method, EPC requires high-tech equipment. However, it offers a more precise and controlled freezing process with the possibility to programme temperature changes and thus avoid the formation of intracellular ice crystals.

In this study, the viability rates of frozen fibroblasts from Tobet dogs with EPC were analysed and compared with those of NEC. The cryoprotectants DMSO and EG were also used in the experiment and the Planer Kryo-330 software freezer was utilised. The possibilities of using this equipment allow you to program the freezing mode and maintain the freezing temperature with an error of 0.01 degrees Celsius.

During the experiment, it was revealed that the viability rate of fibroblasts from Tobet dogs using various cryoprojectors: 10% DMSO and 10% EG using slow program freezing EPC was 60.7 ± 1.1% and 75.7 ± 0.9%, respectively (Table 2), which was higher than when using NEC (Figure 3). Statistical analysis by Student did not reveal significant differences ($P > 0.05$). As in the previous case, the viability of thawed fibroblasts from Tobet dogs was higher when 10% EG was used than when 10% DMSO was used.

Table 2 – Effect of various cryoprotectants and the use of slow program freezing EPC on the viability of fibroblasts of the Tobet breed

Cryoprotector/freezing mode	Relative number of living cells before freezing, %	Relative number of living cells after thawing, %
DMSO/EPC	91.7 ± 0.6a	60.7 ± 1.1b
EG/ EPC	91.7 ± 0.6a	75.7 ± 0.9c
Note, ab, bc P > 0.05		

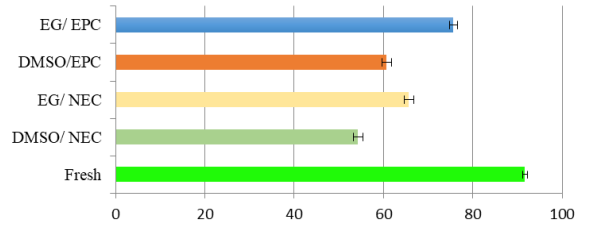

Figure 3 – Viability of fibroblasts from dogs of the Tobet breed when using various cryoprotectants and different freezing methods

The development of methods and the study of the characteristics of the cryopreservation of somatic cells of Tobet breed dogs have allowed us to reach the following conclusions and methodological recommendations that can be used for a more effective conservation of the genetic resources of the Tobet breed:

- when comparing the effectiveness of the two culture media used (RPMI-1640 and DMEM), preference was given to DMEM. When cultured in this medium, the release of fibroblasts from the explant was observed several days earlier, and their growth rate was also noticeably higher;
- in our studies, the use of only one enzyme, a warm 0.25% trypsin solution, turned out to be effective and sufficient for almost complete removal of the fibroblast monolayer from the surface of the culture dish. The optimal incubation time with trypsin was 7-10 minutes at 37 °C;
- during the studies, it was revealed that the most optimal cryoprotector when using slow freezing methods is EG;
- it was found that the use of a software freezer for slow freezing of fibroblasts from Tobet dogs is the most effective.

Conclusion

In this study, the methods were optimised and the properties of cryopreservation of somatic cells (fibroblasts) from Tobet's dogs isolated from skin explants were investigated. The influence of *in vitro* cultivation on their viability was investigated. Their viability was determined during equilibrium and non-equilibrium cryopreservation using different cryoprotectants. Based on the investigations, the most effective approaches were proposed and the first cryobank with somatic cells (fibroblasts) from Tobet's dogs was established. The establishment of the first Tobet dog somatic cell cryobank is a huge step towards the preservation of this unique breed. The results of this study are groundbreaking for similar endeavours as they demonstrate the integration of traditional breeding values with cutting-edge biotechnological approaches.

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