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Motility and CASA parameters of thawed sperm of the Tobet canine breed after vitrification

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Abstract

Dogs of the unique and endangered indigenous national breed Tobet are on the verge of extinction. The cryopreservation of sperm via slow freezing and vitrification may be an effective approach to save these dogs. In this study, the effectiveness of different freezing rates for spermatozoa cryopreservation was examined. Ejaculates were obtained from 16 dogs of the Tobet breed. One part was frozen via slow freezing in nitrogen vapor at heights of 4 cm, 5 cm, and 6 cm above the liquid nitrogen level for 5-6 minutes, and the other part was subjected to programmed freezing in Slow Freezing Rate (SFR) and Fast Freezing Rate (FFR) modes. Vitrification was carried out by adding sucrose at different volumes and BSA to the vitrification solution. The results showed that during slow freezing, the most optimal cryoprotectant/freezing rate combination in nitrogen vapor was freezing 5 cm above the liquid nitrogen level, but the most optimal method was the use of a programmed freezer with SFR and FFR. Additionally, using a vitrification solution (sucrose 0.2 M + 1% BSA) without adding penetrating cryoprotectants also allowed for effective preservation of important sperm parameters and the successful cryopreservation of sperm from dogs of the Tobet breed.

Keywords: Animal biodiversity, Cryopreservation, Cryoprotectants, Spermatozoa, Tobet dog breed, Vitrification.

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Transparency: The authors confirm that the manuscript is an honest, accurate, and transparent account of the study; that no vital features of the study have been omitted; and that any discrepancies from the study as planned have been explained. This study followed all ethical practices during writing.
Institutional Review Board Statement: The animal study protocol was approved by the bioethics committee of the RSE at the REM "Research Institute of Molecular Biology and Biochemistry", named after M.A. Aitkhozhin, CS MSHE of the Republic of Kazakhstan (Protocol No. 1, dated August 18, 2023).
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1. Introduction

The preservation of animal biodiversity is of great relevance and importance. This is due to the decrease in the number and, accordingly, the genetic diversity of local and indigenous animal breeds, which are important linking components in the evolutionary chain. The irreversible loss of animal biodiversity leads to the erosion of genetic material because local and indigenous breeds of animals, formed over many centuries in specific environmental conditions, are characterized by highly adaptive qualities and are resistant to many infectious and invasive diseases. They perform their functions under minimally comfortable conditions; for example, dogs of the Tobet breed are irreplaceable assistants to shepherds.

Dogs of this endangered indigenous breed have been loyal friends and protectors of the Kazakh people for many years, but today, they are on the verge of extinction. Hundreds of years of the Kazakh people's selection and breeding of these ideally strong and hardy dogs, who are reliable guards, may be in vain.

Currently, work on preserving, developing, and restoring national Kazakh dog breeds, including the Tobet, is supported by the Government of the Republic of Kazakhstan. The success of these efforts will depend on selection, which should be based on modern scientific research. At present, the arsenal of selection methods has significantly expanded due to breakthrough achievements and new approaches in genetics, genomics, cytogenetics, and assisted reproductive technologies. Work is underway to assess genetic diversity, study the genetic structure of the Tobet, and analyze its phylogenetic relationships with other dog breeds [1] cryopreserve fibroblasts, and create a biobank of Tobet dogs' germplasm [2].

The cryoconservation of sperm and its subsequent use in artificial insemination and the creation of a biobank will become a promising strategy for preserving this endangered breed. The effectiveness of various cryopreservation methods for protecting valuable livestock breeds and rare species of wild animals has been demonstrated in some studies [3, 4]. As is known, studies of the theoretical aspects of the cryobiology of canine sperm have been conducted in different years [5]. Studies are being conducted to examine different freezing methods and the use of varying components in cryoprotectors, equilibration, and thawing procedures. The cryopreservation of spermatozoa is a method of assisted reproductive biotechnology that increases the possibility of using spermatozoa from the most genetically valuable individuals in selection and also enhances the reproductive capacity of male organisms [6]. Two types of sperm cryopreservation have been developed: cryopreservation and vitrification. Cryopreservation uses a slow, gradual freezing method and results in moderate sperm quality after thawing [7]. On the other hand, vitrification involves ultra-fast freezing of the liquid along with the object, and they pass into a glassy state, thereby avoiding the formation of ice crystals and reducing osmotic shock [8-10]. This method is widely used for the cryopreservation of embryos, oocytes, and tissues [11] in recent years, it has been successfully developed for the cryopreservation of spermatozoa in some animal species. However, little research has been conducted on dogs so far [12-17]. Sperm vitrification methods require improvement, optimization, and standardization to enhance sperm viability after thawing.

In the process of sperm cryopreservation, the use of cryoprotectants is mandatory to minimize cryodamage to spermatozoa [18]. Currently, two groups of cryoprotectants are used: penetrating and non-penetrating. Most often, glycerol is added to cryoprotectants as a penetrating cryoprotectant, which prevents the formation of large intracellular ice crystals, but it has been proven to be toxic to cells [19, 20]. Various combinations of carbohydrates (sucrose, lactose, and trehalose) and proteins (bovine serum albumin, milk, lecithin, and egg yolk) are used as non-penetrating cryoprotectants [21], which prevent the formation of intracellular or extracellular ice crystals by significantly increasing the viscosity of the extender [22]. Egg yolk has been widely used in the cryopreservation of mammalian sperm to protect it from initial cold shock [23, 24]. However, in recent years, there has been a trend toward using non-animal-derived components in extenders to avoid restrictions on the worldwide transfer of sperm due to the risk of prion contamination of samples. Various sperm cryoprotectants have been studied and developed to protect spermatozoa from several factors [25] and the selection of optimal cryoprotectants is an important part of sperm cryopreservation [26, 27]. Currently, commercial cryoprotectants are available for the cryopreservation of canine sperm, which consist of combinations of various chemical compounds. Numerous natural extracts and infusions with cryopreservation properties have also been studied and may be useful as alternative sources of cryoprotectants for the preservation of animal sperm [28]. These substances have high antioxidant capacity, which protects sperm from oxidative damage during freezing [29]. For example, coconut water is used as a natural buffer solution and a biological compound in sperm extenders [30-34] it is isotonic, non-toxic, cheap, effective, and easy to use [30] has highly antioxidant properties [35, 36][and contains many phytohormones [37] sugars, vitamins, electrolytes, and amino acids [38]. Coconut water-based cryoprotectants, with the addition of non-penetrating cryoprotectants, have also been used for the preservation of canine sperm during rapid cryopreservation [17].

Thus, cryopreservation via slow freezing and vitrification may be an effective way to preserve dogs' sperm function, particularly for the Tobet breed. Moreover, the use of sucrose as the main component of extenders during vitrification helps to protect the integrity of the sperm acrosome better than glucose, fructose, or lactose [39]. The scientific aspects of cryopreservation of canine somatic cells, oocytes, spermatozoa, and reproductive tissues remain insufficiently studied and are a priority in the field of canine germplasm cryobiology. The aim of this study was to investigate the potential of different freezing rates and cryoprotectants for effectively protecting spermatozoa to maintain their viability after freezing and thawing.

2. Materials and Methods

2.1. Animals

The objects of this research were 16 dogs of the Tobet breed, from which 2-4 ejaculates were obtained on different days. To conduct this study, approval was obtained from the bioethics committee of the RSE at the REM "Research

Institute of Molecular Biology and Biochemistry," named after [17] CS MSHE of the Republic of Kazakhstan (Protocol No. 1, dated August 18, 2023). This study adhered to the "Bioethical Rules for Conducting Research on Humans and Animals" and complied with the legislation of the Republic of Kazakhstan and the European Convention on Bioethics. During this study, no experiments were conducted on animals; only biomaterial obtained from dogs using a non-invasive procedure that did not cause harm was used. Sperm was collected through manual manipulation as described by Linde-Forsberg and Forsberg [40] in the presence of a teaser bitch in heat. The ejaculate was collected in a prewarmed (36-38^{0}C) glass tube.

2.2. Semen Evaluation

2.2.1. Motility

The motility of fresh and frozen semen was assessed. The sperm concentration and motility parameters were evaluated using a Hamilton Thorne Sperm Analyzer, version IVOS 12.3 (HTR-IVOS 12.3). This CASA system consists of a phase-contrast microscope, camera, minitherm heating stage, image digitizer, and computer for saving and analyzing the data. The following parameters were measured: concentration (CONC), the percentage of motile spermatozoa (MOT), the percentage of spermatozoa with progressive motility (PMOT), the velocity average pathway (VAP), the velocity straight line (VSL), velocity curvilinearity (VCL), amplitude lateral head (ALH), beat cross frequency (BCF), straightness (STR), linearity (LIN), and the average size in square microns of all sperm heads (Area).

The study of these parameters was necessary to assess the quality of the sperm, since in further work on cryopreservation, only semen samples that met the following requirement were used: the percentage of motile spermatozoa ≥60%.

2.3. Reagents

All reagents used in this study were manufactured by Sigma-Aldrich (St.Louis, USA) and Merck SA (St.Louis, USA).

2.4. Extender and Cryoprotectants Used for Cryopreservation of Canine Spermatozoa

Sperm samples were centrifuged at 700g for 10 minutes. The supernatant was removed, and the pellet was then diluted with standard Tris citrate fructose (TCF) extender to a concentration of 2 x 10^8 sperm/mL (first dilution). Extender (Tris–citrate fructose, TCF): Tris, 200 mM; citric acid, 67 mM; fructose, 44.4 mM. Samples were incubated at room temperature (20°C) for 30 minutes and then cooled at 5°C for 60 minutes.

The following cryoprotectants were used in our sperm cryopreservation studies:

Cryoprotector (CPA): Tris, 200 mM; citric acid, 67 mM; fructose, 44.4 mM; egg yolk, 20%; glycerol, 3%; 1% Equex paste (Minitube of America, Verona, WI).

2.5. Nonequilibrium (Nitrogen Vapor) Cryoconservation of Dog Spermatozoa

After cooling, the samples were diluted with the studied cryoprotectants at a ratio of 1:1 (second dilution) and equilibrated at a temperature of 5°C for 15 minutes. The samples were placed in 0.5 ml straws (CryoBiosystem, France) and frozen using different modes at heights of 4 cm (NEC/4), 5 cm (NEC/5), and 6 cm (NEC/6) above the liquid nitrogen level for 5-6 minutes. Using this method, the following cooling modes were achieved (Figure 1).

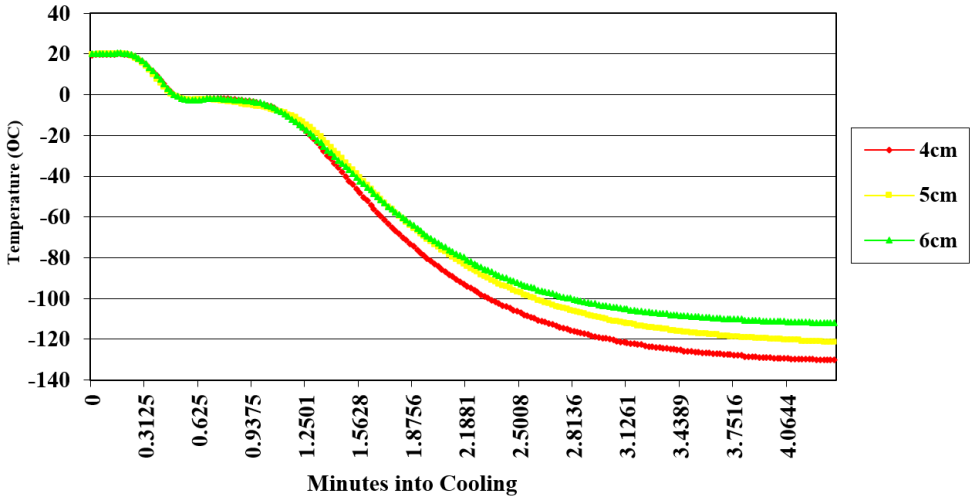

Figure 1.
Cooling rates in 0.5ml straws at 4, 5, and 6 cm above liquid nitrogen.

The thawing of frozen samples was performed in a water bath at 70°C for 5 sec Nöthling and Shuttleworth [41], and the frozen–thawed spermatozoa were analyzed.

2.6. Equilibrium Cryoconservation (Programmed Freezing) of Dog Spermatozoa

Two freezing modes were used for the equilibrium cryopreservation (programmed freezing) of canine spermatozoa:

1. Slow Freezing Rate (SFR): cooling from 5°C to -6°C at a rate of 3°C per minute; siding at -6°C for 1 minute; and cooling from -6°C to -40°C at a rate of 10°C per minute, and from -40°C to -196°C at a rate of 50°C per minute.

2. Fast Freezing Rate (FFR): cooling from 5°C to -6°C at a rate of 3°C per minute; siding at -6°C for 1 minute; and cooling from -6°C to -40°C at a rate of 50°C per minute, then from -40°C to -196°C at a rate of 50°C per minute.

To compare thawing modes, the samples were variously defrosted at different temperatures: in a water bath at 38ºC for 1 min (EQC/SFR 38, EQC/FFR 38) and in a water bath at 70ºC for 10 sec (EQC/SFR 70, EQC/FFR 70).

2.7. Vitrification of Canine Sperm and Thawing of Vitrified Samples

2.7.1. Vitrification Solutions (VSs)

The following vitrification solutions were used in the experiment:

1. HTF (human tubular fluid) (Control);
2. HTF–BSA (bovine serum albumin, BSA, 1%);
3. HTF–BSA + sucrose 0.1M;
4. HTF–BSA + sucrose 0.2M;
5. HTF–BSA + sucrose 0.3M.

2.7.2. Vitrification of Canine Sperm

After adding the vitrification solution, the samples were equilibrated at 5°C for 30 min. The vitrification process was carried out using a slightly modified protocol previously described by Isachenko et al. [42]. Briefly, a foam box was loaded with LN_2 into which, for 5 cm, a metal strainer was immersed. The strainer allows for easy collection of the spheres. Using a micropipette (10–100 µl micropipette, Eppendorf, Hamburg, Germany) held at an angle of 45^0 and at a distance of 11 cm from the LN_2 surface, 30 µl aliquots of spermatozoa suspension (different vitrification solutions) were dropped directly into the LN_2. Upon contact with the LN_2, a sphere immediately forms and floats to the surface. After about 4-6 seconds, the sphere solidifies and falls to the bottom of the strainer. Once this process has taken place, the procedure is repeated to obtain more spheres. It is important to wait for the sphere to precipitate before adding another microdrop, because they can stick together and form a larger drop. After solidification, the spheres can be easily collected with the use of a small spoon. The solid spheres (30-40 pieces) were packaged into 2.0 ml cryovials and stored in LN_2 for at least a week before thawing for further evaluation.

Thawing was performed by quickly immersing spheres, one at a time (with no more than five spheres), in 5 ml of HTF–BSA 1%, preheated to 37°C, via gentle shaking for 5–10 seconds. The thawed sperm suspension was incubated at 37°C and 5% CO_2 for 10 minutes and then centrifuged at 300 g for 5 minutes. The cell pellet was finally resuspended in 50 µl HTF for sperm evaluation only.

2.8. Statistical Analysis

Values were expressed as mean ± standard deviation (SD), and for statistical analysis of the obtained data, nonparametric analysis of variance (Kruskal–Wallis) was used with a significance level of $P < 0.05$. Differences between groups were established using Dunn's multiple comparison test [43].

3. Results

3.1. Sperm Collection and CASA Analysis

Over the course of this study, 63 ejaculates were collected from 16 fertile dogs with offspring.

3.2. Effect of Nonequilibrium (Nitrogen Vapor) Cryopreservation on the Motility and CASA parameters of Canine Spermatozoa

In this research, we examined the effect of nonequilibrium cryopreservation (in liquid nitrogen vapor) on the motility and CASA parameters of canine spermatozoa. One cryoprotectant and three freezing modes were used. The empirical identification of the most effective cryoprotectant/freezing regime pair remains the most applicable method in scientific practice for sperm cryopreservation. Thus, in this research, the samples were divided into three experimental groups based on the principle of analogs. The results obtained from the study of the CASA parameters of frozen–thawed canine spermatozoa using various cryoprotectants and different freezing modes revealed that the most optimal cryoprotectant/freezing mode combination was the following:

- NEC/5 cm (total motility 50.41 ± 9.37%; progressive motility 43.07 ± 4.98%) (Table 1, Figure 2).

Figure 2 shows significant parameters affecting the motility of the frozen–thawed spermatozoa of Tobet dogs, such as total motility (MOT) and progressive motility (PR) during cryopreservation using different freezing modes.

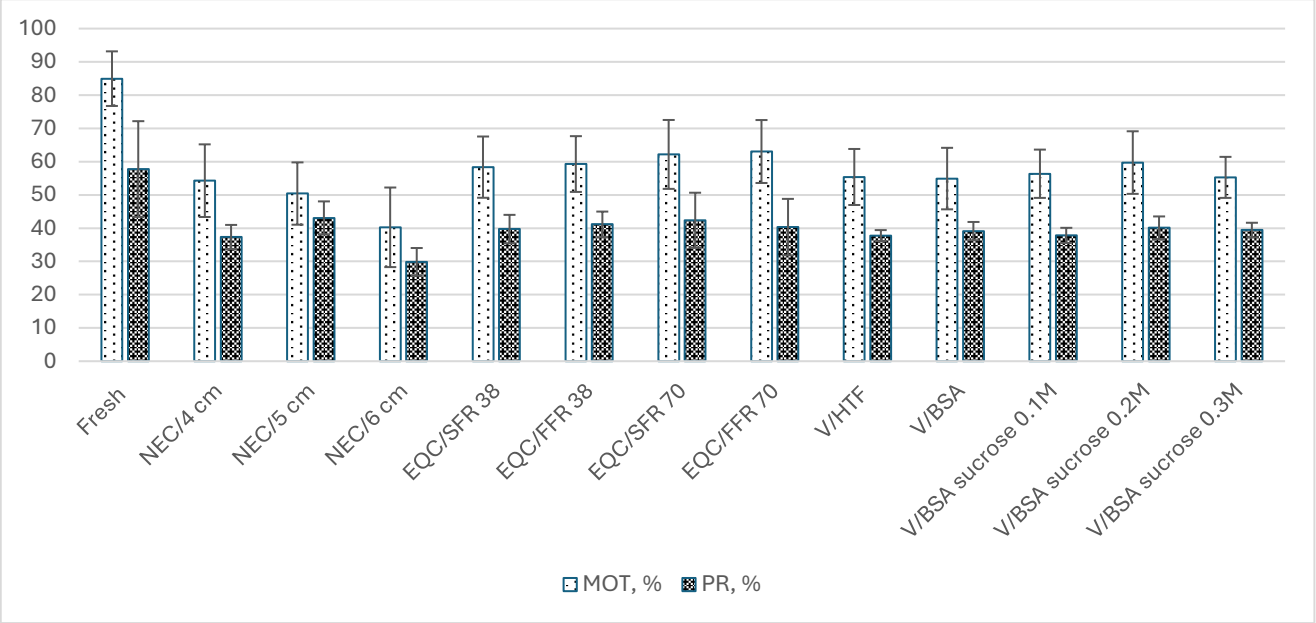

Figure 2.
Motility parameters of frozen–thawed dog spermatozoa after different freezing regimens. MOT total motility; PR progressive motility.

Table 1.
Motility and CASA parameters of frozen–thawed dog spermatozoa measured by IVOS analyzer after nonequilibrium (nitrogen vapor) cryoconservation.

	Fresh	NEC/4 cm	%	NEC/5 cm	%	NEC/6 cm	%
MOT	84.95 ± 8.21[a.]	54.28 ± 10.92[b]	-36.10	50.41 ± 9.37[b]	-40.66	40.26 ± 11.96[b]	-52.61
PR	57.78 ± 14.39	37.35 ± 3.63	-35.36	43.07 ± 4.98	-25.46	29.86 ± 4.17	-48.32
VAP	151.39 ± 12.12	115.55 ± 3.89	-21.15	107.74 ± 4.05	-26.48	57.37 ± 10.39	-60.85
VSL	131.35 ± 15.24	101.53 ± 4.12	-19.24	98.73 ± 3.95	-21.47	62.58 ± 12.16	-50.22
VCL	221.59 ± 15.39	150.56 ± 5.21	-25.72	144.38 ± 6.32	-28.76	90.64 ± 10.31	-55.28
ALH	5.39 ± 1.15	5.95 ± 0.78	4.57	4.27 ± 1.35	-24.96	7.26 ± 2.37	27.59
BCF	24.25 ± 3.39	11.58 ± 1.11	-47.77	12.29 ± 1.31	-44.56	7.84 ± 2.75	-64.64
STR	85.26 ± 6.49	85.83 ± 1.25	1.74	83.29 ± 2.67	-1.27	65.39 ± 9.67	-22.49
LIN	67.31 ± 9.19	66.53 ± 1.34	3.71	62.83 ± 1.92	-2.06	66.57 ± 12.19	3.77
Area	4.79 ± 2.26	5.57 ± 1.95	13.21	5.59 ± 2.03	13.62	5.61 ± 2.12	14.02

Note: ab $P < 0.05$.

Explanation of abbreviations: MOT total motility; PR progressive motility; VAP average velocity of the smoothed cell path; VSL average velocity measured in a straight line; VCL average velocity; ALH amplitude lateral head; BCF beat cross frequency; STR straightness; LIN linearity; Area average size in square microns of all sperm heads; % difference from the fresh sample.

The percentage indicators of the motility parameters of Tobet dogs' frozen–thawed spermatozoa and their differences relative to a fresh sample after nonequilibrium cryopreservation in nitrogen vapor are shown in the diagram below (Figure 3).

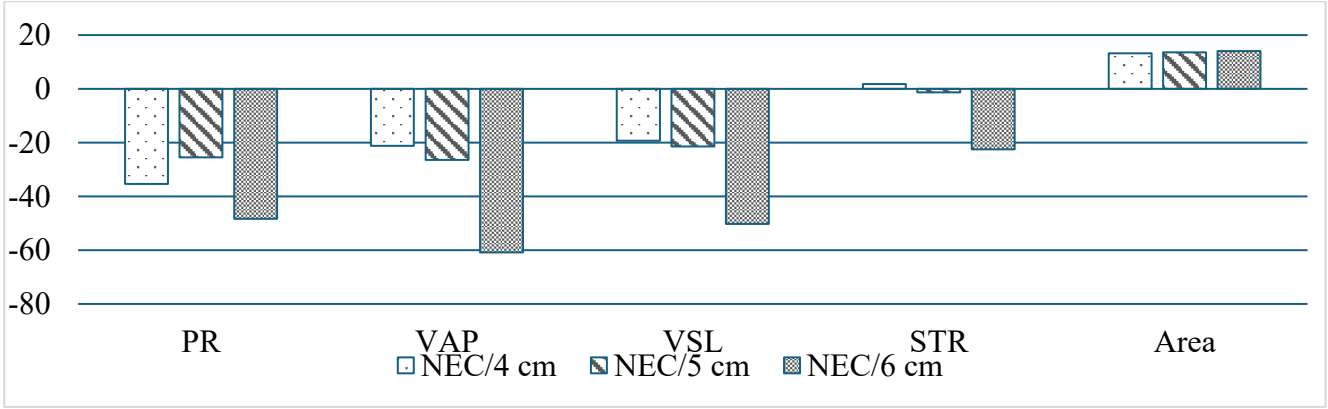

Figure 3.
Percentage differences in the motility parameters of frozen–thawed canine spermatozoa from a fresh sample after nonequilibrium (nitrogen vapor) cryopreservation. PR progressive motility; VAP average velocity of the smoothed cell path; VSL average velocity measured in a straight line; STR straightness; Area average size in square microns of all sperm heads.

It should be noted that the cryoprotectant was previously developed and has a long history of use in the cryopreservation of canine sperm. Statistical analysis of total motility showed significant differences between groups ($P < 0.05$).

3.3. Studying the Effect of Equilibrium Cryopreservation (Programmed Freezing) on the Motility and CASA parameters of Canine Spermatozoa

While conducting studies on the effect of equilibrium cryopreservation (programmed freezing) on the motility and CASA parameters of canine spermatozoa, two freezing modes and two thawing modes were used.

The results obtained from the study of the CASA parameters of frozen–thawed canine spermatozoa using various cryoprotectants and different freezing modes revealed that the most optimal cryoprotectant, freezing mode, and thawing mode combinations were as follows:

- EQC /FFR 38 (total motility 59.28 ± 8.38%; progressive motility 41.17 ± 3.82%);
- EQC /SFR 70 (total motility 62.17 ± 10.36%; progressive motility 42.39 ± 8.27%);
- EQC/FFR 70 (total motility 63.05 ± 9.46%; progressive motility 40.33 ± 8.48%) (Table 2).

Table 2.
Motility parameters of frozen–thawed dog spermatozoa measured by the IVOS analyzer after equilibrium (programmed) cryoconservation.

	Fresh	EQC/SFR 38	%	EQC/FFR 38	%	EQC/SFR 70	%	EQC/FFR 70	%
MOT	84.95 ± 8.21[a]	58.35 ± 9.21[b]	-31.31	59.28 ± 8.38[b]	-30.22	62.17 ± 10.36[b]	-25.78	63.05 ± 9.46[b]	-25.78
PR	57.78 ± 14.39[c]	39.72 ± 4.28[d]	-31.26	41.17 ± 3.82[d]	-28.75	42.39 ± 8.27[d]	-30.20	40.33 ± 8.48[d]	-30.20
VAP	151.39 ± 12.12	110.39 ± 3.89	-24.67	117.35 ± 3.53	-19.92	122.24 ± 4.57	-17.57	120.79 ± 9.19	-17.57
VSL	131.35 ± 15.24	111.39 ± 4.12	-11.40	119.39 ± 4.36	-5.03	123.74 ± 5.83	-2.25	122.89 ± 9.03	-2.25
VCL	221.59 ± 15.39	167.62 ± 7.49	-17.30	175.38 ± 6.96	-13.47	174.43 ± 7.49	-11.19	179.99 ± 9.07	-11.19
ALH	5.39 ± 1.15	6.39 ± 1.15	12.30	6.57 ± 2.12	8.79	6.19 ± 1.62	12.83	6.42 ± 1.84	12.83
BCF	24.25 ± 3.39	17.49 ± 2.53	-21.11	18.75 ± 3.58	-15.43	19.85 ± 3.29	-9.11	20.15 ± 2.79	-9.11
STR	85.26 ± 6.49	82.49 ± 4.28	-2.22	83.12 ± 5.39	-1.47	85.38 ± 7.18	1.99	86.04 ± 9.47	1.99
LIN	67.31 ± 9.19	65.43 ± 3.57	2.00	66.27 ± 5.49	3.30	66.39 ± 6.27	2.53	65.77 ± 7.24	2.53
Area	4.79 ± 2.26	5.62 ± 1.99	14.23	5.64 ± 1.48	14.63	5.61 ± 2.13	14.02	5.59 ± 1.55	13.62

Note: ab, cd $P > 0.05$.

Explanation of abbreviations: MOT total motility; PR progressive motility; VAP average velocity of the smoothed cell path; VSL average velocity measured in a straight line; VCL average velocity; ALH amplitude lateral head; BCF beat cross frequency; STR straightness; LIN linearity; Area average size in square microns of all sperm heads; %—% difference from the fresh sample.

Figure 4 shows the percentage indicators of the motility parameters of Tobet dogs' frozen–thawed spermatozoa and their differences relative to a fresh sample after equilibrium cryopreservation with programmed freezing.

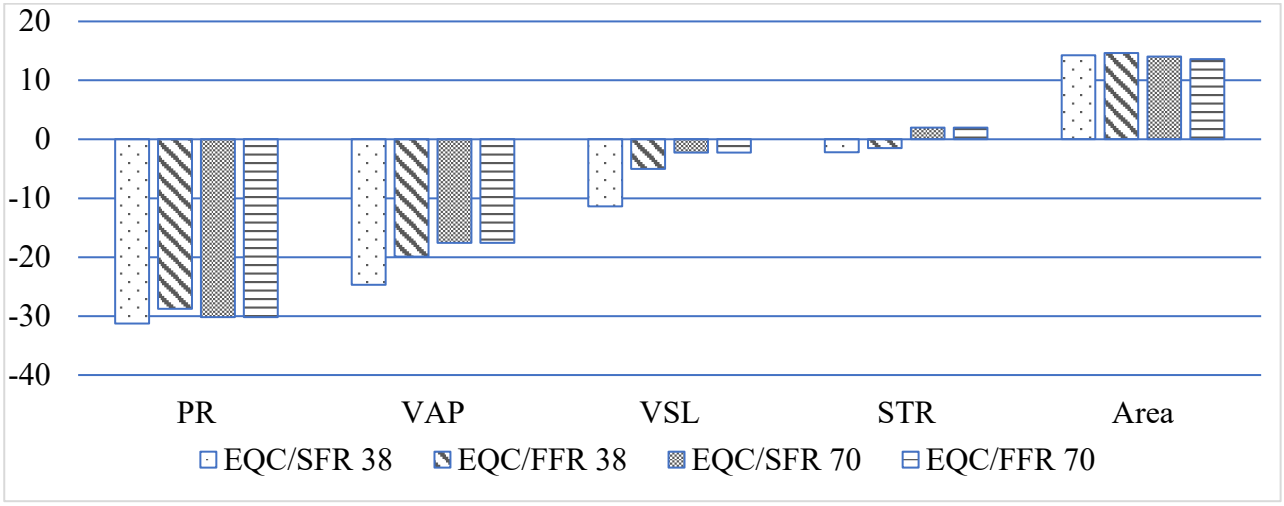

Figure 4.
Percentage differences in the motility parameters of frozen–thawed canine spermatozoa from a fresh sample after equilibrium (programmed) cryopreservation. PR progressive motility; VAP average velocity of the smoothed cell path; VSL average velocity measured in a straight line; STR straightness; Area average size in square microns of all sperm heads.

Statistical analysis of motility and progressive motility did not reveal any significant differences between the experimental groups ($P > 0.05$).

Thus, it can be assumed that the use of a cryoprotectant and SFR and FFR freezing modes involving a programmed freezer is the most optimal method for the equilibrium cryopreservation of canine spermatozoa.

Table 3.
Motilility parameters of frozen–thawed dog spermatozoa measured by the IVOS analyzer after vitrification.

	Fresh	HTF	%	BSA	%	BSA + Sucrose 0.1M	%	BSA + Sucrose 0.2M	%	BSA + Sucrose 0.3M	%
MOT	84.95 ± 8.21[a]	55.39 ± 8.42[b]	-34.80	54.91 ± 9.25[b]	-35.36	56.36 ± 7.26[b]	-33.66	59.72 ± 9.41	-29.70	55.27 ± 6.17[b]	-34.94
PR	57.78 ± 14.39[c]	37.78 ± 1.64[d]	-34.61	39.12 ± 2.75[d]	-32.29	37.83 ± 2.27[d]	-34.53	40.14 ± 3.39[d]	-30.53	39.45 ± 2.18[d]	-31.72
VAP	151.39 ± 12.12	85.27 ± 4.23	-41.81	83.19 ± 3.93	-43.23	93.23 ± 4.01	-36.38	101.17 ± 4.48	-30.96	115.57 ± 3.21	-21.13
VSL	131.35 ± 15.24	93.61 ± 3.14	-25.54	95.28 ± 2.85	-24.21	96.29 ± 2.14	-23.41	99.98 ± 7.37	-20.47	97.47 ± 2.28	-22.47
VCL	221.59 ± 15.39	117.44 ± 9.69	-42.06	109.61 ± 10.84	-45.92	123.39 ± 9.31	-39.12	139.28 ± 11.35	-31.28	119.78 ± 7.73	-40.90
ALH	5.39 ± 1.15	5.39 ± 1.15	-5.27	6.85 ± 2.27	20.39	6.37 ± 3.11	11.95	7.26 ± 3.26	27.59	5.94 ± 2.82	4.39
BCF	24.25 ± 3.39	24.25 ± 3.39	9.38	28.74 ± 4.18	29.63	29.07 ± 3.87	31.12	31.81 ± 2.94	43.48	27.96 ± 2.27	26.12
STR	85.26 ± 6.49	71.06 ± 0.94	-15.77	73.83 ± 1.28	-12.48	79.28 ± 1.11	-6.02	79.62 ± 5.18	-5.62	77.47 ± 1.81	-8.17
LIN	67.31 ± 9.19	32.16 ± 6.52	-49.87	53.56 ± 5.73	-44.57	49.42 ± 4.39	-22.96	56.52 ± 10.17	-11.89	51.89 ± 5.73	-19.11
Area	4.79 ± 2.26	5.29 ± 1.27	10.44	5.38 ± 1.65	12.32	4.99 ± 1.73	4.18	4.96 ± 1.36	3.55	4.92 ± 2.19	2.71

Note: ab $P < 0.05$ cd $P > 0.05$.
Explanation of abbreviations: MOT total motility; PR progressive motility; VAP average velocity of the smoothed cell path; VSL average velocity measured in a straight line; VCL average velocity; ALH—amplitude lateral head; BCF—beat cross frequency; STR straightness; LIN linearity; Area average size in square microns of all sperm heads; %—% difference from the fresh sample.

3.4. Studying the Effect of Vitrification on the Motility and CASA parameters of Canine Spermatozoa

During the study of the effect of vitrification on the motility and CASA parameters of canine spermatozoa, five vitrification solutions (VSs) were used. Thus, the samples were divided into five experimental groups based on the analog principle.

The results obtained from the study of the CASA parameters of vitrified–thawed canine spermatozoa using various vitrification solutions revealed that the most optimal VS was BSA + sucrose 0.2M (total motility, 59.72 ± 9.41%; progressive motility, 40.14 ± 3.39%) (Table 3).

The results of the studies on the motility parameters of Tobet dogs' frozen–thawed spermatozoa after vitrification are presented in Figure 5, which shows the percentage indicators of the motility parameters and their differences relative to those of a fresh sample.

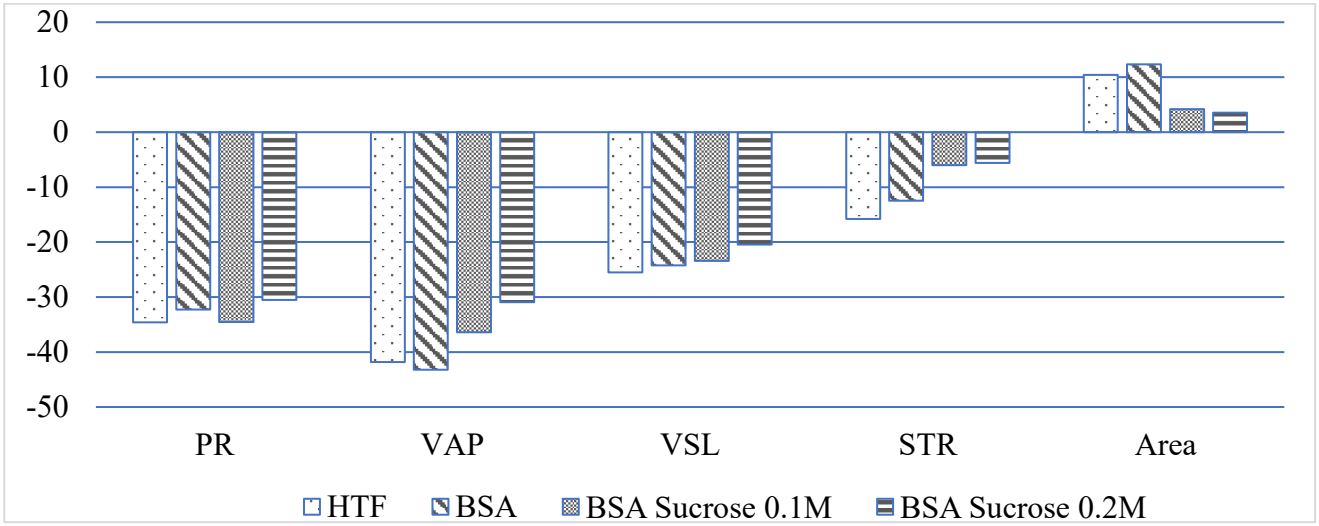

Figure 5.
Percentage differences in the motility parameters of frozen–thawed canine spermatozoa from a fresh sample after vitrification. PR progressive motility; VAP average velocity of the smoothed cell path; VSL average velocity measured in a straight line; STR straightness; Area average size in square microns of all sperm heads.

Analysis of the total motility index showed a statistically significant difference ($P < 0.05$), while analysis of progressive motility revealed no significant differences between the groups ($P > 0.05$).

Thus, it can be assumed that the use of the solution BSA + sucrose 0.2M in the vitrification of canine spermatozoa is the most optimal.

4. Discussion

In recent years, cryobiological research on sperm has focused on studying various aspects of conventional freezing. It is known that the effectiveness of cryopreservation methods is based on the use of both penetrating and non-penetrating cryoprotectants. The widespread application of penetrating cryoprotectants to different types of cells is associated with their properties, such as rapid permeability through the cytoplasmic membrane, along with their toxicity. These properties are directly related to damage to cells during osmotic shock when they are saturated with these cryoprotectants before and during freezing, as well as when the cryoprotectants are removed after thawing [44]. The importance of non-penetrating cryoprotectants in the composition of cryoprotectors is associated with their auxiliary action. For example, the presence of sugars in ethylene glycol-based cryoprotectors [45] leads to a decrease in the toxicity of these permeable cryoprotectants and makes it possible to reduce the concentration of penetrating cryoprotectants used, which is necessary for the effective binding of intracellular water [46]. In general, the inclusion of osmotically active, non-penetrating compounds in vitrifying solutions leads to both additional cell dehydration and the stabilization of intracellular and extracellular osmotic pressure. Thus, non-penetrating cryoprotective sugars have a unique property: the stabilization of the cell membrane [47-49].

An alternative method of cryopreservation is vitrification, whose aim is to completely prevent the formation of ice crystals by forming a vitreous body using ultra-rapid freezing rates Luyet [50]. Fahy, et al. [51] found that vitrification does not allow the formation of disulfide bridges between protein molecules, which cause protein denaturation. The main feature of vitrification is its rapid execution, and it does not require the purchase of expensive equipment.

Although the method has been successfully applied to oocytes and embryos, it cannot be applied to spermatozoa, since vitrification solutions use high concentrations of penetrating cryoprotectants, which have a toxic effect. As is known, the shapes and sizes of sperm heads are factors that determine their cryosensitivity [52]. Accordingly, the greatest cold damage is observed in sperm with a certain morphology, with a large blade-shaped and flat head. Mouse sperm is very sensitive to cold damage compared to sperm from other species. It was found that mouse spermatozoa do not tolerate osmotic shock during equilibration in and the removal of cryoprotectants Songsasen and Leibo [53]. Nakagata and Takeshima [54] employed only the impermeable cryoprotectant carbohydrate raffinose without the use of penetrating cryoprotectants for the cryopreservation of mouse spermatozoa.

As is known, integral membrane proteins are located in the bilipid layer, and it is possible that their properties can change, especially those that function as transport channels for calcium absorption. For example, cooling increases the permeability of these channels, which affects calcium regulation [55, 56]. This has important implications for sperm function and viability [57].

Based on this context, we decided to investigate the effect of different freezing rates on the motility and CASA parameters of the frozen–thawed spermatozoa of Tobet dogs. During our studies, it was established that with nonequilibrium (nitrogen vapor) cryopreservation, the most optimal results were obtained using the freezing method at a height of 5 cm from the LN_2 level. The MOT was 50.41 ± 9.37%, and the progressive motility (PR) was 43.07 ± 4.98%. When using equilibrium (programmed) cryopreservation, the optimal combination was slow-freezing cryoconservation/thawing at $70^{O}C$; the MOT was 62.17 ± 10.36% and the progressive motility (PR) was 42.39 ± 8.27%. Additionally, after the application of fast-freezing cryoconservation/thawing at $70^{O}C$, the MOT was 63.05 ± 9.46% and the progressive motility (PR) was 40.33 ± 8.48%. When studying the effect of different concentrations of sucrose during vitrification, it was found that the most effective vitrification solution was the one containing 1% BSA + 0.2M sucrose. The MOT was 59.72 ± 9.41% and the progressive motility (PR) was 40.14 ± 3.39%.

In studying the CASA parameters of frozen–thawed spermatozoa, attention was paid to the following parameters: progressive motility, VAP, VSL, STR, and Area. This was due to the fact that the algorithm for determining the relative number of spermatozoa with progressive motility in this research was calculated based on the setup for canine sperm specified by the equipment manufacturer: VAP = 100 μ/s and STR = 75% (STR = (VSL/VAP) X 100). Another indicator of cryopreservation efficiency was the Area parameter (average size in square microns of all sperm heads), studied before and after freezing. The results are shown in Tables 1, 2, and 3. It should be noted that a relative change in the average area of sperm heads was observed in all of the above-mentioned studied groups, including those involving vitrification and the use of BSA 1% + 0.2M sucrose.

The obtained research results reveal that vitrification without the use of penetrating cryoprotectants allows for the successful cryopreservation of Tobet dogs' sperm. The use of a solution (sucrose 0.2 M + 1% BSA) in the vitrification of canine sperm can effectively preserve important parameters, such as total motility, progressive motility, and the average head area of spermatozoa, as well as their CASA parameters.

Thus, due to the simplicity, the speed of the implementation, and the low cost of the protocol, sperm cryopreservation via vitrification is the most effective method tested. In addition, it does not require the use of special, expensive cryobiological equipment, which greatly facilitates its use in laboratories.

5. Conclusion

The research results showed that with nonequilibrium (nitrogen vapor) cryopreservation, the most optimal outcomes were obtained using the freezing method at a height of 5 cm from the liquid nitrogen level. When using equilibrium (programmed) cryopreservation, the slow-freezing cryoconservation/thawing at $70^{\circ C}$ and fast-freezing cryoconservation/thawing at $70^{\circ C}$ modes showed good results. When studying the effect of various solutions during vitrification, it was found that the most effective vitrification solution was that containing BSA 1% + 0.2 M sucrose. Comparing the efficiency of freezing modes, it should be noted that when using a vitrification solution containing 1% BSA and 0.2 M sucrose as cryoprotectants, vitrification without the use of penetrating cryoprotectants avoids toxicity and can provide high-quality results close to those of traditional freezing methods, and it can be useful as an alternative to these in the conventional cryopreservation of canine sperm. Given that settlements are located at large distances from each other across Kazakhstan, and dogs may be in remote areas very far from the locations of research centers, it is difficult to deliver dogs and their material to scientific centers for investigation. Considering the above, it can be assumed that vitrification of Tobet canine spermatozoa is an optimal method for short-term preservation based on motility parameters, which is consistent with the experimental results obtained, and allows freezing the material on site. There is also the prospect of using vitrified spermatozoa of Tobet dogs in assisted reproduction technologies (artificial insemination, intrauterine insemination).

In addition, vitrification does not require special, expensive cryobiological equipment due to its simple protocol, speed of execution, and low cost.

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