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UDC 636.082.12:573.6.086.83 *Original Article*

VIABILITY OF CANINE'S OVARIAN TISSUE AFTER NONEQUILIBRIUM CRYOCONSERVATION

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ABSTRACT

Modern approaches to reproductive biotechnology include the development of effective methods for long-term storage of gametes and reproductive tissues of animals. One of the promising areas is cryopreservation of ovarian tissue, which allows preserving fertility, especially in rare and valuable species. However, the effectiveness of this technology directly depends on the type of cryoprotectant, freezing mode, cooling rate and storage method. Nonequilibrium cryopreservation is a method in which biological samples are exposed to liquid nitrogen vapor without strict software control of cooling. This approach allows for a simpler procedure but requires careful optimization of conditions to preserve tissue viability. The aim of this work is to evaluate the effect of various cryoprotectants and the altitude of the samples (4, 5 and 6 cm above the surface of liquid nitrogen) on the morphological preservation of follicles in the ovarian tissue of dogs after nonequilibrium cryopreservation. A study was conducted to investigate the effect of nonequilibrium cryopreservation in liquid nitrogen vapor on the morphofunctional state of ovarian tissue in dogs. Four cryoprotectors (dimethyl sulfoxide, ethylene glycol, propylene glycol, glycerol) and three temperature regimes (4 cm, 5 cm and 6 cm from the liquid nitrogen level) were used. After thawing, a histological analysis was performed to assess the degree of preservation of different types of follicles. It was found that the best viability indicators were observed when using 1.5 M dimethyl sulfoxide (4 and 6 cm), glycerol (4 cm) and propylene glycol (5 cm). The data obtained are important for the development of effective protocols for cryopreservation of ovarian tissue in dogs in order to preserve the gene pool and reproductive potential.

Key words: ovarian tissue, dogs, cryopreservation, cryoprotectants, liquid nitrogen vapor, follicles.

INTRODUCTION

Based on the provisions of the International Convention on Biological Diversity and in accordance with the Interlaken Declaration (ID) on Animal Genetic Resources, one of the priority tasks formulated by these documents is "the conservation, sustainable use and inventory of the genetic resources of living organisms". Priority objects of protection in agrobiocenoses should be varieties of cultivated plants and local breeds of domesticated animals [1]. In this case, local aboriginal breeds are considered cultural heritage. This is due to the fact that local animal breeds, formed in specific environmental conditions, are characterized by high adaptive qualities, they are resistant to many diseases.

In recent years, Kazakhstan has faced the threat of irreversible loss of some valuable animal populations of the domestic gene pool. The loss of breed diversity is not only the loss of unique and invaluable genetic diversity, but also a narrowing of genetic potential, leading to a decrease in allelic diversity. All developed countries have created national programs for the conservation, reproduction and study of domestic animal breeds. In our republic, work on preserving biodiversity is carried out only through efforts to preserve populations of rare animal species in the wild. However, the possibility of preserving their genetic material should not be neglected. The creation of genetic cryobanks is extremely important for preserving the genetic material of animals with potentially high economic value or for using the population of endangered species. Gene banking can be actively used to increase population sizes and minimize inbreeding, to provide breeds and alleles for any use in the longer term, such as re-establishing a lost breed and restoring genetic diversity within a breed to healthy levels [2-6]. Conservation of genetic resources of endangered species can be carried out *in situ* (natural habitat or zoos and breeding facilities) or *ex situ in vitro* by cryopreservation of reproductive organs (ovarian tissue), gametes (sperm, mature and immature oocytes) and embryos.

Today, biotechnological methods such as artificial insemination and embryo transfer are well developed and used in breeding and conservation programs. Artificial insemination (AI) is one of the important and frequently used procedures in breeding. From a small number of selected males, it is possible to obtain sufficient sperm to inseminate thousands of females per year. However, artificial insemination and embryo transplantation are not sufficient to prevent inbreeding if the supply of gametes is limited. To solve the problem of inbreeding, it is necessary to create a gene bank of male and female gametes and embryos, collecting them from a large number of individuals. An alternative to storing embryos is cryopreservation of mature oocytes, but the use of this technique has some difficulties associated with cryopreservation of mature oocytes, which have a large volume of cell cytoplasm, a high level of metabolism and chromosomes in the metaphase of meiosis II. Mature oocytes are very sensitive to cooling. Cryopreservation of oocytes at the metaphase II stage of meiosis gives disappointing results due to the problems encountered during fertilization and embryonic development. Cryopreservation is accompanied by compaction of the zona pellucida as a result of premature release of cortical granules from the oocyte cytoplasm and damage to the cy-

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toskeleton [7, 8]. Freezing at the germinal vesicle stage has been proposed as an alternative to freezing at the metaphase II stage for cryopreservation of oocytes. At the germinal vesicle stage, there is no sensitive spindle apparatus that can be damaged. However, here too, strengthening of the zona pellucida and damage to the cytoskeleton were observed [9]. In addition, cryopreservation of oocytes at the germinal vesicle stage is more problematic in terms of survival and maturation of oocytes to the metaphase II stage. Until a reliable method for *in vitro* oocyte maturation is developed, cryopreservation of oocytes at the germinal vesicle stage is not advisable. Additionally, ovarian stimulation is required to obtain oocytes and embryos, which usually takes some time depending on the animal species. These methods are not acceptable in emergency cases, and the smaller quantities of oocytes obtained *in vivo* do not guarantee maximum preservation of genetic material. Limited success with the methods described above has focused research into cryopreservation of immature oocytes in primordial follicles, which are located in the cortex of the ovary, which would be very useful in preserving female gametes in maximum quantity. Because mammalian ovaries contain thousands of eggs enclosed in follicles, they represent 90% of the follicular population. This method allows the storage of hundreds of immature oocytes *in situ in vitro* without the need for ovulation induction. Cryopreservation of ovarian tissue has more advantages and the probable success of this method can be explained by the following: the oocyte is less differentiated, has a small size, is in the cellular division stage (prophase of the 1st meiotic division), has low metabolic activity, lacks a transparent zona, a monolayer of granulosa cells, cortical granules do not exist and the follicle is less sensitive to ischemia [10].

Cryopreservation of ovarian tissue can be performed using two main methods: slow freezing and vitrification. Slow programmed freezing (or “slow freeze”) consists of a gradual programmed decrease in temperature [11,12,13]. Prevention of intracellular ice formation and, accordingly, preservation of cells is achieved by slowly lowering the temperature, which leads to a gradual release of water and its replacement with cryoprotectants after preliminary exposure in an equilibration solution. The second type of slow freezing, unbalanced cryopreservation, is a method in which biological samples are exposed to liquid nitrogen vapor without software control, using a polystyrene foam block with cryotube cells.

The first attempts made in the 1950s to freeze rodent ovarian tissue in the presence of glycerol at –79°C were not very successful [14, 15], since the temperature reduction was not controlled. Over the next 20 years, the results of work related to the cryopreservation of ovarian tissue did not find practical application. Later, Candy et al. [16] showed that approximately 20% of follicles survived in ovarian tissue frozen in the presence of glycerol, but using a more precise cooling rate. The development and introduction of new cryoprotective agents (PROH, EG and DMSO) into cryobiological practice in the 1970s marked the beginning of a new stage of research in this area. The fundamental protocol for low-temperature preservation in the presence of DMSO for sheep ovarian tissue was developed by Gosden R.G. and his co-authors in 1994. Numerous experimental data obtained by Gook D.A. and his co-authors [17,18] demonstrated successful freezing of ovarian tissue fragments in the presence of PROH, where the percentage of follicle viability was more than 50%. Later studies conducted by Hovatta O. and his co-authors in 1996 [19] demonstrated the resistance of human ovarian tissue to low-temperature preservation in the presence of DMSO or in a combination of PROH and sucrose. The resistance of ovarian tissue to cryodamage and the high level of preservation of primordial stage follicles were confirmed by other researchers [20-22].

Current trends in ovarian tissue cryopreservation include optimization of both freeze/thaw protocols and culture technology to preserve the maximum pool of follicles in order to increase the number of viable follicles after cryopreservation. In this regard, it is important to optimize the size of tissue fragments, select and determine the concentration of the cryoprotectant, the time and temperature of exposure to the tissue, as well as develop new freezing/thawing modes and improve existing methods of cryopreservation of ovarian tissue for wide application in the field of preserving the genetic resources of endangered animal species and in clinical practice. At the same time, the development of cryogenic technology for preserving animal genomes promotes international exchange of genetic material, creation of a bank of their gene pool, and optimal cryopreservation methods achieved in work with mammals can be used as a model in medicine.

Therefore, the aim of this research work is to develop optimal cryopreservation methods for preserving ovarian tissue of local dog breeds of Kazakhstan in order to preserve and reproduce their gene pool.

MATERIALS AND METHODS

Research materials

Ovarian tissue samples for cryopreservation

Ovarian tissues of female dogs of various breeds, which were ovariectomized in a veterinary clinic, were used for the experiments.

The study was approved by the bioethics commission 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).

Ovarian tissue collection and transportation for the experiment

For the entire experiment, ovaries were transported to the laboratory at 37°C in DPBS with 5% FCS or L-15 with 5% FCS (basic media). Ovaries were freed from ligaments and washed for 30 sec in the basic medium with the addition of antibiotics 100 μg/ml penicillin-streptomycin. They were then placed in DPBS with 5% FCS, the brain portion was removed, leaving the cortex portion containing a small fragment of the medulla, and they were divided into small fragments measuring 1.5-2.0 x 1.0-1.5 x 1.0-1.2 mm using a disposable scalpel. From each ovary, 1-2 fragments were sent for histology as a control.

Slow freezing of ovarian tissue in liquid nitrogen vapor

17 ovaries were placed in L-15 with 5% FCS, the brain part was removed, leaving the cortex containing a small fragment of the medulla, they were divided into small fragments

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measuring 1.5-2.0 x 1.0-1.5 x 1.0-1.2 mm using a disposable scalpel. The samples obtained in the amount of 132 fragments were divided into 12 experimental groups according to the principle of analogues:

1) Dimethyl sulfoxide/4cm (DMSO/4);

2) Ethylene glycol/4cm (EG/4);

3) Propanediol/4cm (PROH/4);

4) Glycerin/4cm (GL/4);

5) Dimethyl sulfoxide /5cm (DMSO/5);

6) Ethylene glycol /5cm (EG/5);

7) Propanediol /5cm (PROH/5);

8) Glycerin /5cm (GL/5);

9) Dimethyl sulfoxide /6cm (DMSO/6);

10) Ethylene glycol /6cm (EG/6);

11) Propanediol /6cm (PROH/6);

12) Glycerin /6cm (GL/6).

To equilibrate the samples, different concentrations of cryoprotectants were prepared: 0.3 M, 0.75 M, 1.5 M CPA with 0.12 M, 0.25 M, and 0.5 M Sucrose in DPBS. A three-stage introduction of cryoprotectants was used in all experimental groups:

CPA (1.5 M DMSO; 1.5 M PROH; 1.5 M EG; 1.5 M GL):

1) in DPBS + 0.3 M CPA + 0.12 M Sucrose were equilibrated for 5 min.;

2) in DPBS + 0.75 M CPA + 0.25 M Sucrose were equilibrated for 5 min.;

2) in DPBS + 1.5 M CPA + 0.5 M Sucrose were equilibrated for 10 min.

Straws (CBS, CryoBioSystem, France) with a capacity of 0.5 cm^3 were used for freezing the samples. Filling and sealing of the marked straws was carried out for 10 minutes before freezing. During this time, the samples were equilibrated (3rd stage of introducing the cryoprotectant) in the thawing solution. The time interval from placing the sample in the medium with the cryoprotectant until the start of cooling did not exceed 10-20 minutes. After the introduction of cryoprotectors, freezing of the experimental groups was carried out in 3 different modes: freezing in nitrogen vapor at a height of 4 cm, 5 cm and 6 cm from the surface for 20 minutes, then immersed in liquid nitrogen and stored in Dewar vessels (Fig. 1).

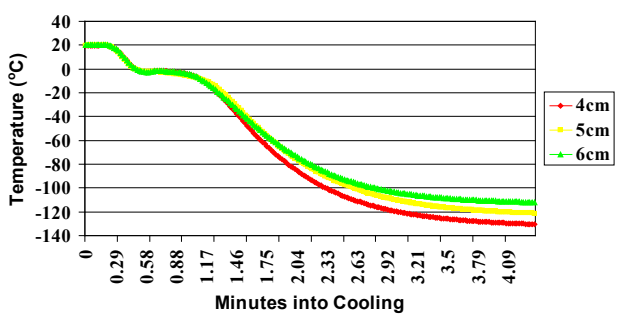

Figure 1 - Diagram of the temperature regime of freezing in nitrogen vapor at a height of 4 cm, 5 cm and 6 cm.

After thawing, the samples were fixed in a 10% neutral formalin saline solution, histological preparations were made, and microscopic examination was performed.

Thawing of ovarian tissue in all experiments

For thawing, frozen samples in cryostraws were kept in atmospheric air at room temperature for 30 seconds. They were then immersed in a 100°C boiling water bath and kept there for 60 sec. The exposure time in the water bath was visually monitored by the presence of ice in the test tube; once the ice was thinned to 1-2 mm, the test tube was removed from the boiling water, and eventually the temperature of the environment inside the test tube was between 4 and 10 °C.

Histological analysis

Fresh and cryopreserved ovarian tissue fragments were fixed in 10% neutral formalin saline for 24 hours, dehydrated and embedded in paraffin. Sections of 5 μm thickness were made on a sled microtome, stained with hematoxylin and eosin and histologically examined under a microscope at objective magnifications of x20 and x40. As a result, 52 paraffin blocks and 156 histological preparations were prepared, from which 439 microtome sections were obtained. The quality of the ovarian tissue was assessed based on the morphological picture of the follicles. The number of viable and damaged cells was counted. Histological sections were analyzed, examining only follicles with a visible nucleus to exclude duplicate counting of the same follicle in the analyzed section. The morphology of the follicles was identified according to the classification of K. Oktay, modified according to Gougeon [23]: primordial - the oocyte is surrounded by one layer of flattened granulosa cells; primary - the oocyte is surrounded by a single layer of cuboidal granulosa cells; secondary - the oocyte is surrounded by more than two layers of granulosa cells located on the basal membrane, around which there are single theca cells; antral - the oocyte is enlarged in volume, surrounded by several layers of granulosa cells, a cavity containing follicular fluid is formed. Microphotography was performed using a Zeiss Axiostarplus microscope, "Videotest Morphology".

Statistical data processing

Microsoft Excel was used for statistical processing. The difference was considered statistically significant at $p<0.05$ according to the Student's t-test.

RESULTS

Study of the effect of various cryoprotectants and different freezing modes on the histological structure of canine ovarian tissue

Preantral follicles were well preserved in all control samples. When using the cryoprotectant dimethyl sulfoxide (DMSO/4), some preantral follicles were preserved. Many preantral follicles were in a state of destruction. In early antral follicles, damage to granulosa cells was observed, but the oocyte in it retained its structural organization (Fig. 2 a, b, c).

When using glycerol (GL/4), significant amounts of both primordial and subsequent stages of follicular growth are present. As can be seen from the figures (2 d, e, f), along with damaged early antral follicles, small antral follicles (730.55 μm and 599.82 μm in size, oocyte 100.0 μm, 98.0 μm in size) are found, the normal structure of which is well preserved. The oocyte is surrounded by a crown of cumulus cells arranged in several layers and a clearly defined zona pellucida. The ooplasm contains numerous cortical granules and several yolk inclusions (Fig. 2 f).

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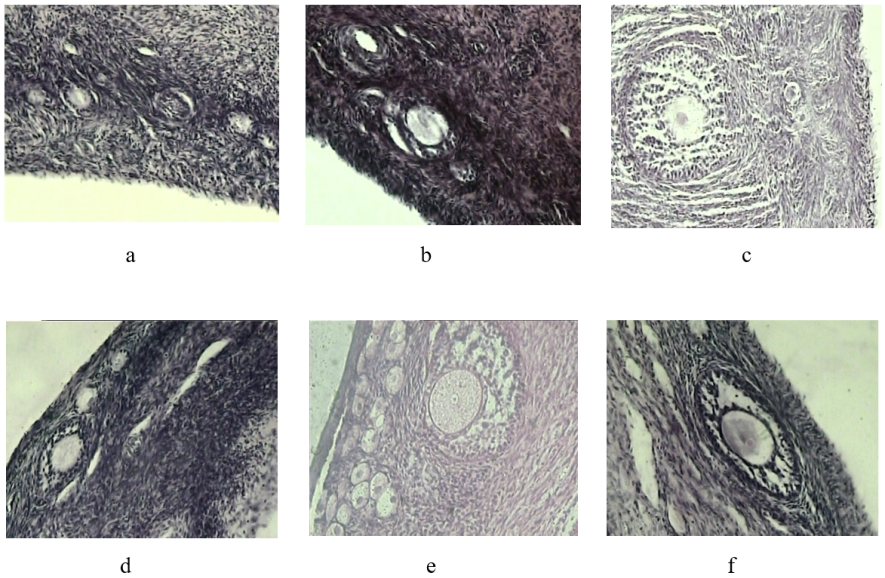


a – DMSO/4: preserved primordial follicles; b – DMSO/4: preserved primary and secondary follicles; c – DMSO/4: early antral follicle preserved; d – GL/4: primordial follicles preserved; e – GL/4: preserved primordial and secondary follicles; f – GL/4: early antral follicle preserved. Staining according to G-E and VG. Magnification x200, x400.

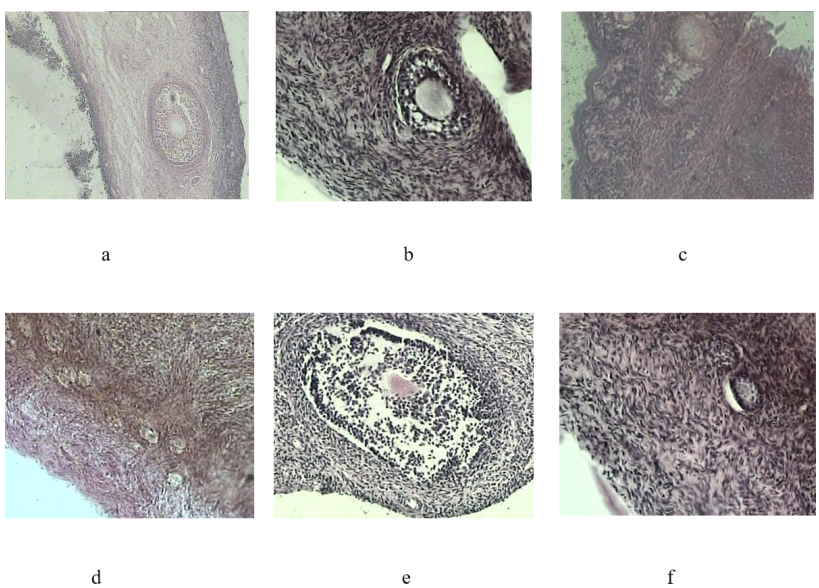
Figure 2 - Histological sections of normal follicles after freezing in liquid nitrogen vapor at a distance of 4 cm.

The use of ethylene glycol and propylene glycol (EG/4 and PRON/4) as cryoprotectants did not give good results. Damage to follicles was noted.

Microscopic analysis of the DMSO/5, GL/5, EG/5 and PROH/5 groups showed that the preantral follicles were well preserved in the PROH/5 group (Fig. 3b). Here, the number of preantral follicles that retained the usual structural organization, as well as early and small antral follicles, was relatively significant. In the EG/5 group, the primordial and antral follicles were in a state of destruction. When glycerol (GL/5) was used as a cryoprotectant, damage to the preantral follicles was observed (Fig. 3c).

The study showed that when using dimethyl sulfoxide (DMSO/5), only individual antral and primordial follicles retained their normal structural organization. In the preserved antral follicles, the oocyte was spherical in shape, had fine-grained cytoplasm, a clearly defined zona pellucida, almost completely surrounded by a crown of cumulosa cells (Fig. 3a).

Microscopic examination revealed that when using DMSO/6, along with damaged primordial and early antral follicles, both early antral and primordial follicles retained their normal structural organization (Fig. 3e). In the remaining groups GL/6, EG/6 and PROH/6, partial preservation of preantral follicles was observed, but the follicles were mainly destroyed (Fig. 3d, f).



a – DMSO/5: preserved antral follicle with healthy oocyte; b – PRON/5: early antral follicle is preserved; c – GL/5: primordial follicles are damaged; d – EG/6: primordial follicles are preserved; e – DMSO/6: partial damage to early antral follicle; f – PRON/6: damaged primordial follicles. Staining according to GE and van Gieson. Magnification x200, x400.

Figure 3 - Histological sections of normal follicles after freezing in liquid nitrogen vapor at a distance of 5 and 6 cm.

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Table 1 - Relative number (%) of morphologically normal follicles in frozen dog ovary tissue using various cryoprotectants and various freezing modes.

Freezing modes	DMSO	EG	PROH	GL
4 cm	61,2±4,1[a]	–	–	67,2±2,5[a]
5 cm	48,5±4,9[b]	–	70,2±5,6[b]	–
6 cm	55,2±3,9[c]	–	–	38,1±5,1[c]
Control	97,3±1,2[d]	97,3±1,2[d]	97,3±1,2[d]	97,3±1,2[d]
Note: ad, bd, cd P>0.05				

The data presented in Table 1 show that the use of 1.5 M GL, 1.5 M DMSO (4 cm) and 1.5 M PROH (5 cm), as well as 1.5 M DMSO (6 cm) as a cryoprotectant, has a positive effect on the viability of ovarian follicles in the tissue. Student-Fisher statistical analysis revealed significant differences in the results obtained in all groups compared to the results of the control group (P ad,bd,cd> 0.05). However, statistical analysis did not reveal any significant differences between the groups (P<0.05).

CONCLUSION

The results of the study showed that the morphofunctional state of ovarian tissue in dogs after nonequilibrium freezing varies significantly depending on the cryoprotectant used and the cooling regime. The highest degree of preservation of morphologically normal follicles was observed with the use of 1.5 M dimethyl sulfoxide (at heights of 4 and 6 cm), glycerol (4 cm) and propylene glycol (5 cm). At the same time, cryoprotectors ethylene glycol and propylene glycol demonstrated low efficiency at other heights.

These results highlight the importance of choosing both the optimal cryoprotectant and the height of sample placement in liquid nitrogen vapors during nonequilibrium cryopreservation. The proposed conditions can be used in developing standard protocols for freezing canine ovarian tissue for biobanking, transplantology, and gene pool preservation.

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ВЫЖИВАЕМОСТЬ ОВАРИАЛЬНОЙ ТКАНИ СОБАК ПОСЛЕ НЕУРАВНОВЕШЕННОЙ КРИОКОНСЕРВАЦИИ

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АННОТАЦИЯ

Современные подходы к репродуктивной биотехнологии включают разработку эффективных методов длительного хранения гамет и репродуктивных тканей животных. Одним из перспективных направлений является криоконсервация овариальной ткани, что позволяет сохранить фертильность, особенно у редких и ценных видов. Однако эффективность этой технологии напрямую зависит от типа криопротектора, режима замораживания, скорости охлаждения и способа хранения.

Неуравновешенная криоконсервация — метод, при котором биологические образцы подвергаются воздействию паров жидкого азота без строгого программного контроля охлаждения. Этот подход позволяет упростить процедуру, но требует тщательной оптимизации условий, чтобы сохранить жизнеспособность ткани.

Целью настоящей работы является оценка влияния различных криопротекторов и высотного положения образцов (4, 5 и 6 см над поверхностью жидкого азота) на морфологическую сохранность фолликулов в овариальной ткани собак после неуравновешенной криоконсервации. Проведено исследование по изучению влияния неуравновешенной криоконсервации в парах жидкого азота на морфофункциональное состояние овариальной ткани собак. Использованы четыре криопротектора (диметилсульфоксид, этиленгликоль, пропиленгликоль, глицерин) и три температурных режима (4 см, 5 см и 6 см от уровня жидкого азота). После размораживания выполнен гистологический анализ, направленный на оценку степени сохранности различных типов фолликулов. Установлено, что наилучшие показатели жизнеспособности наблюдаются при применении 1,5 М диметилсульфоксида (4 и 6 см), глицерина (4 см) и пропиленгликоля (5 см). Полученные данные имеют значение для разработки эффективных протоколов криоконсервации овариальной ткани у собак с целью сохранения генофонда и репродуктивного потенциала.

***Ключевые слова**:* овариальная ткань, собаки, криоконсервация, криопротекторы, пары жидкого азота, фолликулы

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ТЕҢГЕРІМСІЗ КРИОКОНСЕРВАЦИЯДАН КЕЙІН ИТТЕРДІҢ АНАЛЫҚ БЕЗІ ТІНІНІҢ ӨМІР СҮРУІ

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АҢДАТПА

Репродуктивті биотехнологияның заманауи тәсілдері жануарлардың гаметаларын және репродуктивті ұлпаларын ұзақ уақыт сақтаудың тиімді әдістерін жасауды қамтиды. Перспективалы бағыттардың бірі аналық без тінін криоконсервациялау болып табылады, бұл әсіресе сирек және бағалы түрлердің фертильділігін сақтауға мүмкіндік береді. Дегенмен, бұл технологияның тиімділігі криопротектор түріне, мұздату режиміне, салқындату жылдамдығына және сақтау әдісіне тікелей байланысты.

Теңестірілмеген криоконсервация - бұл биологиялық үлгілерді салқындатуды қатаң бағдарламалық бақылаусыз сұйық азот буының әсеріне ұшырату әдісі. Бұл тәсіл процедураны жеңілдетуге мүмкіндік береді, бірақ тіндердің өміршеңдігін сақтау үшін жағдайларды мұқият оңтайландыруды талап етеді.

Бұл жұмыстың мақсаты әртүрлі криопротекторлардың және үлгілердің биіктігінің (сұйық азоттың бетінен 4, 5 және 6 см) теңгерілмеген криоконсервациядан кейін иттердің аналық безінің ұлпасында фолликулалардың морфологиялық сақталуына әсерін бағалау болып табылады. Сұйық азот буындағы теңгерімсіз криоконсервацияның иттердегі аналық без тінінің морфофункционалды күйіне әсерін зерттеу үшін зерттеу жүргізілді. Төрт криопротектор (диметилсульфоксид, этиленгликоль, пропиленгликоль, глицерин) және үш температура режимі (сұйық азот деңгейінен

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4 см, 5 см және 6 см) қолданылды. Жібіткеннен кейін фолликулалардың әртүрлі түрлерінің сақталу дәрежесін бағалау үшін гистологиялық талдау жүргізілді. Ең жақсы өміршеңдік көрсеткіштері 1,5 М диметил сульфоксиді (4 және 6 см), глицерин (4 см) және пропиленгликоль (5 см) қолданғанда байқалатыны анықталды. Алынған деректер генофондты және репродуктивті әлеуетті сақтау мақсатында иттердің аналық безі тінін криоконсервациялаудың тиімді хаттамаларын жасау үшін маңызды.

Негізгі сөздер: аналық без ұлпасы, иттер, криоконсервация, криопротекторлар, сұйық азот буы, фолликулалар